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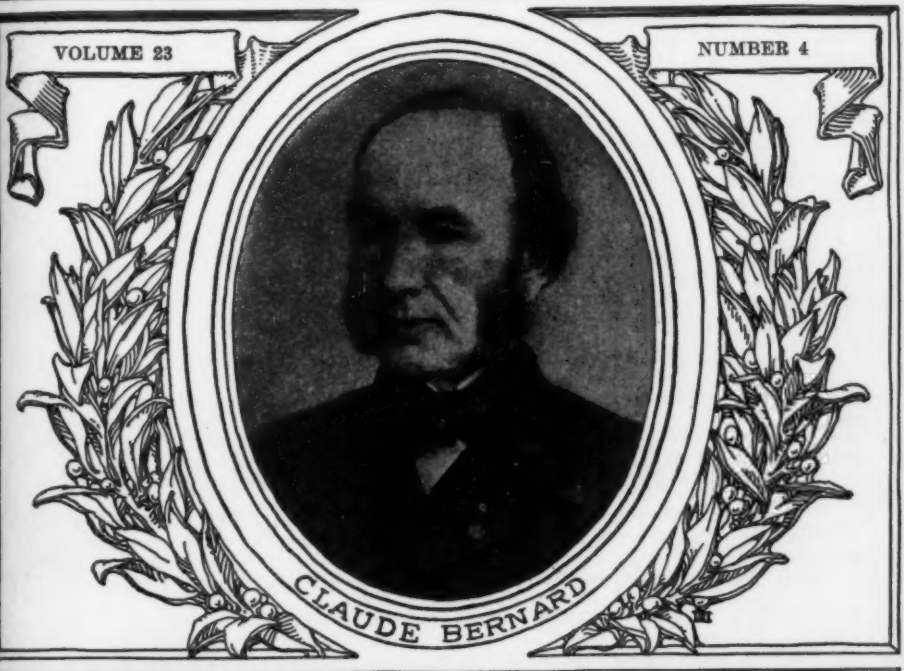

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CONGENITAL MALFORMATIONS INDUCED IN RATS BY MATERNAL NUTRITIONAL DEFICIENCY

JOSEF WARKANY AND ROSE C. NELSON

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ONE PLATE (FOUR FIGURES)

(Received for publication October 29, 1941)

In previous communications ('40, '41) we reported that congenital malformations appeared in the offspring of externally normal albino rats of the Sprague-Dawley strain when the females were reared and bred on a deficient diet. On external inspection the defective offspring frequently showed shortening of the mandible and protrusion of the tongue, shortening and distortion of the extremities and various degrees of syndactylism of the hands and feet; thinness of the abdominal wall in the ventral midline, protrusion of abdominal viscera, reduction in size of the uro-genital papilla and defects of the eyes also were observed in rare cases. After the specimens were cleared by the Schultze-Dawson method it was found that certain bones were often abnormal while other bones apparently were never affected. Thus, in spite of great variations, a certain pattern of skeletal malformations was recognized in the offspring of the females bred on this deficient diet. This pattern which has been described in detail previously (Warkany and Nelson, '41) will be referred to as "pattern of diet I" in the following paragraphs. Figures 1 and 2 illustrate an abnormal animal in its external appearance and an abnormal cleared specimen respectively. In the present publication we wish to present further data on the nutritional aspects of the experiments.

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Breeding results on diet I

The deficient diet referred to is essentially the Steenbock and Black diet no. 2965 ('25) supplemented with viosterol to forestall the development of rickets. This diet which will be referred to as "diet I" in the following pages had the following percentage composition: whole yellow corn meal [1] 76, wheat gluten [2] 20, calcium carbonate C.P. 3 and sodium chloride C.P. 1. These ingredients were mixed thoroughly and fed in cups ad libitum. Sixty International Units of vitamin D as viosterol were administered to each female by pipette every tenth day. The females were put on diet I at the age of 4 weeks. They grew slowly and the process of maturation was delayed. However, at the age of 5–6 months they frequently attained a weight of 150 gm. and regular cycles appeared. Such females were bred to males of the same strain that had been reared on the adequate stock diet. When sperm was found in the vaginal smear the females were isolated. The vaginal smears were then continued to make certain the diagnosis of pregnancy which was considered established with the appearance of the blood sign. During the period of expected parturition the pregnant females were watched day and night in order to prevent the mothers from eating their young during or after delivery.

In fifty-nine females on diet I, 122 pregnancies were observed. Ninety-three of these pregnancies were carried through and resulted in the delivery of 484 offspring. These consisted of 295 normal and 189 abnormal young. Of the ninety-three litters observed in this group forty-three litters consisted entirely of normal offspring, thirty-three entirely of abnormal and seventeen of both normal and abnormal offspring. The average litter size on this diet was only 5.20. Abnormal young and fetuses in resorption were frequently found in the same pregnancy (figs. 3 and 4). Of 122 pregnancies twenty-nine were entirely resorbed. This incidence of

[1] Bolted, Tuxedo brand.
[2] Pure Gluten Food Company.

resorptions in females on diet I is very high compared with that found in females on adequate diets. It appears that abnormal fetuses are frequently resorbed before the pregnancy is finished. Of the fifty-nine females on diet I thirty-two were pregnant more than once. The results of these repeated (two to six) pregnancies indicate that in later pregnancies abnormal litters occur more frequently. Eventually all the females on this diet gave birth to one or more abnormal litters, with the exception of two animals which have been bred five times and have as yet not delivered abnormal offspring.

Breeding results on a stock diet

A group of thirty-eight females of the same strain were reared and bred on Bills' modification of Steenbock's stock diet (Bills et al., '28), which had the following percentage composition: yellow corn meal 57, whole milk powder 25, linseed oil meal 12, crude casein 3.7, alfalfa leaf meal 1.5, iodized table salt 0.4, and calcium carbonate 0.4. Ninety-one pregnancies were observed in this group. Only one of these ended in resorption and ninety litters were obtained. These consisted of 614 young, of which 613 were normal and one was abnormal. The latter had a shortened humerus and femur but normal tibia, mandible and ribs; this pattern of abnormalities differs from the pattern of diet I. The average litter size on the stock diet was 6.82.

Breeding results on a modification of diet I with $CaCO_3$ content reduced and 2% liver added (diet II)

While the breeding experiments on diet I were proceeding, twenty-three females of the Sprague-Dawley strain were reared and bred on a diet consisting of: yellow corn meal 78, wheat gluten 18, calcium carbonate C.P. 1, sodium chloride 1 and dried pig liver 2%, a diet devised by Remington ('37) as an "improved goitrogenic diet." In our experiments viosterol was administered to the animals fed this diet as it was to

those fed diet I. The dried pig liver was prepared as recommended by Remington. On this diet fifty-five pregnancies resulting in fifty-three litters were observed. They consisted of 350 young, of which 348 were normal and two abnormal. The two abnormals were not of the pattern of diet I. One animal was dwarfed and tailless, and after clearing showed a fused sternum but no other skeletal defects. The other abnormal young was of normal size and externally the mouth appeared reduced in size, the upper lip showed a double cleft and the tongue did not protrude. After clearing, fusion of both halves of the mandible in the midline and a bifurcation of a rib on each side were seen. The average litter size on diet II was 6.60. Two pregnancies ended in resorption.

Breeding results on a modification of diet I with $CaCO_3$ content reduced and 0.5% liver added (diet VII)

This diet consisted of yellow corn meal 78, wheat gluten 19.5, sodium chloride C.P. 1, calcium carbonate C.P. 1, and dried pig liver 0.5%. Vitamin D was given as in diet I. Forty pregnancies were observed in fourteen females on this diet. They resulted in thirty-seven litters consisting of 236 young. Of these 233 were normal and three abnormal. The three abnormal animals were of the pattern of diet I. The average litter size was 6.37. Three pregnancies ended in resorption.

While the females on diet I had 39.05% abnormal offspring, those on diet II had none and those on diet VII had only 1.27% abnormal offspring. Diet II and diet VII differ from diet I essentially in two respects, the addition of liver and the reduction of calcium carbonate. It was thought advisable to test the preventive power of these two factors separately. Therefore two additional diets were devised: Diet VI which is essentially identical with diet I except for the addition of 2% liver, and diet V which is essentially identical with diet I except for the reduction of calcium carbonate from 3% to 1%.

Breeding results on a modification of diet I with 2% liver added (diet VI)

Nineteen females were reared and bred on diet VI which had the following percentage composition: yellow corn meal 76, wheat gluten 18, calcium carbonate C.P. 3, sodium chloride C.P. 1, and pig liver 2. Viosterol was given as in diet I. Fifty-six pregnancies and fifty-one litters were observed in the females on this diet. The offspring consisted of 322 normal and no abnormal young. The average litter size was 6.31. Five pregnancies ended in resorption.

Breeding results on a modification of diet I with $CaCO_3$ content reduced (diet V)

Twenty-four females were reared and bred on diet V which had the following percentage composition: yellow corn meal 78, wheat gluten 20, calcium carbonate C.P. 1, and sodium chloride C.P. 1. Viosterol was given as with diet I. Sixty pregnancies resulting in fifty-five litters occurred in this group. Three hundred and forty-nine young, of which 316 were normal and thirty-three were abnormal, were obtained on this diet. The abnormalities seen externally and after clearing showed the pattern of diet I. The average litter size was 6.35. Five pregnancies ended in resorption.

Breeding results on a modification of diet I with alcoholic liver extract added (diet IV)

An alcoholic extract of liver was made and added to diet I in order to test its preventive capacity.

Dried pig liver as prepared for diet II was extracted with 95% alcohol in a Soxhlet apparatus for 3 days. An amount of extract equivalent to 4 gm. of dried liver was used in making up 100 gm. of this diet. The extract was added to the corn meal and the alcohol evaporated on a steam table.

Ten females on this diet had thirty-seven pregnancies and thirty-five litters. Two hundred and thirty-five young, all normal, were obtained. The average litter size was 6.71. Two pregnancies ended in resorption.

Table 1 summarizes the breeding results on all the diets discussed in this communication. Only abnormal offspring of the pattern of diet I are listed as "abnormal" in this table.

The occurrence of abnormal offspring of the pattern of diet I was prevented when the females were fed diet I supplemented by 2% liver or alcoholic liver extract. A supplement of 0.5% liver did not prevent the abnormalities entirely but reduced their incidence materially. Reduction of the calcium content of diet I also resulted in a reduced incidence of abnormalities.

TABLE 1

Breeding results on different diets.

MOTHER REARED ON	OFFSPRING			ABNORMAL OFFSPRING PER CENT OF TOTAL OFFSPRING
	Total	Normal	Abnormal	
Diet I	484	295	189	39.05
Diet I with $CaCO_3$ content reduced (diet V)	349	316	33	9.46
Diet I with $CaCO_3$ content reduced and 0.5% liver added (diet VII)	236	233	3	1.27
Diet I with $CaCO_3$ content reduced and 2% liver added (diet II)	348	348	0	0
Diet I with 2% liver added (diet VI)	322	322	0	0
Diet I with alcoholic liver extract added (diet IV)	235	235	0	0
Stock diet	613	613	0	0

Breeding results on alternating diets

It seemed of interest to find out whether a female which had been on diet I and had had abnormal offspring would have normal offspring when changed to a liver-containing diet before her next mating and conversely, whether a female which

had had normal offspring while on a liver-containing diet would have abnormal offspring when changed to diet I. The following is a representative experiment of this type.

Female 45 B, reared and bred on diet I, was mated to male 47 R and delivered abnormal offspring. On the day of this delivery she was changed to diet II and then mated to the same male. In the subsequent litter she delivered entirely normal offspring. The breeding on alternating diets was repeated several times, the change always being made on the day of delivery and before the next mating. The results are presented in table 2. In this experiment the same female and

TABLE 2

Breeding results on alternating diets.

	OFFSPRING	
ON DIET	Normal	Abnormal
I	0	4
II	8	0
I	6	1
II	10	0
I	8	2
I	0	7
II	3	0

the same male were mated seven times. As may be seen from the table, alternating the dietary regime of the mother resulted in the production of alternate abnormal and normal litters. In many similar breeding experiments on alternating diets it was found that a change from diet I to the liver-containing diet invariably resulted in normal offspring. However, the change from the liver-containing diet to diet I was sometimes not followed immediately by the appearance of an abnormal litter and only when the females were kept on diet I during subsequent pregnancies did abnormal offspring appear.

Breeding results on diet I obtained with a different strain of rats

The results so far reported were obtained with albino rats of the Sprague-Dawley strain. Through the kindness of Dr. L.

Emmett Holt of the Pediatric Department of Johns Hopkins University we were supplied with a different strain of rats which had been inbred there for many years. This strain was derived originally from Dr. E. V. McCollum's rat colony and it consisted of albino and yellow and black hooded rats.

These rats were put on diet I at the age of 1 or 2 months and bred to males of the same strain as soon as regular cycles were observed. Twelve females had eighteen litters consisting of 123 young of which 48, or 39.02%, were abnormal. Thus the incidence of abnormalities in this strain was equal to that observed in the Sprague-Dawley strain. The congenital defects of the offspring resembled in all respects those described as the pattern of diet I in the Sprague-Dawley strain. Abnormalities were found in albino and in hooded offspring.

DISCUSSION

It has been asserted often that faulty nutrition of the fetus may be a cause of congenital malformations. The faulty nutrition may have its origin in various disturbances. Faulty implantation of the ovum, diseases of the placenta, interruption of the umbilical blood flow, toxic substances transmitted through the placenta and deficiencies in the diet of the mother have been suggested as causes of deformities in the mammalian embryo or fetus. The last-mentioned cause has aroused new interest in recent years. When it was recognized that minute amounts of certain foodstuffs were required for satisfactory nutrition a qualitative malnutrition of the pregnant animal could be suspected to be the cause of a faulty development of the embryo.

Hale ('35) described microphthalmia, cleft palate and other congenital malformations in the offspring of vitamin A-deficient sows. Andersen ('41) reported a high incidence of congenital diaphragmatic hernia in the young of rats bred on a diet deficient in vitamin A. However, Hart and coworkers ('33), Hughes ('34), and Cannon ('40) found no congenital malformations in the offspring of vitamin A-deficient animals.

Moore and coworkers ('35) observed blindness that developed during the intra-uterine period and was associated with a constriction of the optic nerve in calves whose dams had received rations with a poor quality roughage. Micromelia of chicken embryos caused by a nutritional deficiency of the laying hens was reported by Byerly and coworkers ('35).

In our experiments a significant difference was found in the incidence of congenital abnormalities in newborn rats according to the diet of the mother. More than one-third of the offspring of females fed diet I showed skeletal abnormalities of a definite pattern. This pattern did not occur in the offspring of females fed a balanced stock diet. This stock diet differed from diet I in so many respects that it was not possible to draw conclusions as to the identity of the protective factor in the stock diet. Abnormalities of the pattern of diet I were also absent in the offspring of the females bred on diet II. This ration differed from diet I in two respects only — in the addition of 2% pig liver and in the reduction of the calcium carbonate content from 3 to 1%. In order to decide which of these two changed factors prevented the abnormalities, diet VI was devised, differing from diet I only in that 2% liver was added. Since the offspring of the females reared on this diet were also entirely normal, it may be concluded that liver contains a factor which prevents the appearance of the abnormalities of the pattern of diet I. The nature of this preventive factor has not yet been established. However, it seems to be present in pig liver in large amounts, since the addition of 2% of the dried organ proved effective. That the preventive factor in liver can be extracted by alcohol is shown by the results observed with diet IV with which entirely normal offspring were obtained (table 2). The offspring of females bred on diet VII which contained only 0.5% dried liver were not entirely protected. Three of the 236 offspring obtained on this diet showed abnormalities of the pattern of diet I.

The high calcium content of diet I apparently contributed in some way to the high incidence of congenital abnormalities seen in the offspring of females bred on this diet. On diet V

which differs from diet I in only one respect, the reduction of calcium carbonate from 3 to 1%, a significantly lower incidence of congenital malformations was observed.

All of these experimental results are in accord with our assumption that the congenital malformations described are due to a faulty diet of the mother. This assumption is further supported by the observation that females on diet I which had normal offspring in the first litter frequently had abnormal offspring in the second or subsequent litters. It is possible that a store of the protective factor present in the mother at the beginning of the breeding experiment becomes exhausted during one or more pregnancies. The experiments with alternating diets lend further support to the explanation of the malformations on a nutritional basis. When a female which had had abnormal offspring on diet I was changed to the liver-containing diet II and bred again, she always produced entirely normal offspring. The preventive factor apparently becomes effective very rapidly. However, when a female which had had normal offspring on diet II was changed to diet I and was bred again, she had sometimes normal and sometimes abnormal offspring. This behavior can be explained by the assumption that in the first case (normal offspring after changing to diet I) the female had stored a reserve of the preventive factor adequate for this pregnancy, while in the second case (abnormal offspring after changing to diet I) the storage was inadequate.

The fact that identical abnormalities could be obtained under similar dietary conditions in different strains of rats points also to a nutritional basis for the phenomenon described. The experiments reported have given rise to several questions which are under investigation at the present time. An attempt is being made to identify the protective factor in liver or its alcoholic extract. At the same time experiments are being conducted in which various known accessory foodstuffs are being added to diet I in order to test their protective value. An investigation concerned with the critical period of

development in which the abnormalities are determined is also in progress.

SUMMARY

1. Skeletal abnormalities occurred in about one-third of the offspring of female rats reared and bred on Steenbock and Black's rachitogenic diet no. 2965 supplemented with viosterol (diet I).

2. Similar skeletal defects were not found in the offspring of females of the same strain reared and bred on a stock diet.

3. Similarly no such defects were observed in the offspring of females of the same strain reared and bred on diet II, a diet which differs from diet I in that it contains 2% dried pig liver and only 1% of calcium carbonate.

4. The defects were also absent in the offspring of females reared and bred on diet I supplemented by 2% pig liver only (diet VI).

5. If in diet I the calcium carbonate content was reduced from 3 to 1% only about one-tenth of the offspring showed abnormalities of the pattern of diet I.

6. By alternately breeding the same female on diets I and II, abnormal and normal litters could be produced alternately.

7. The same pattern of abnormalities was obtained in the offspring of rats of two different strains reared and bred on diet I.

8. Apparently a nutritional factor that is absent or inadequate in diet I and present in liver in large amounts is necessary for the normal intra-uterine development of the rat.

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PLATE 1

EXPLANATION OF FIGURES

1 Abnormal newborn rat showing receding mandible, protruding tongue, syndactylism of hand and deformed leg.

2 Cleared specimen of an abnormal newborn rat showing shortening of the mandible, absence of the radius, great reduction in size of the ulna, fusion of the ribs, absence of the tibia and reduction in size of the fibula.

3 Ventral view of reproductive tract of a parturient rat reared and bred on diet I. From the left uterine horn an abnormal young had been removed. In the right uterine horn and in the region of the cervix fetuses in resorption may be seen. Several additional implantation sites may be noted in the right uterine horn.

4 A litter of one abnormal young and four fetuses in absorption.

1

2

3

4

THE EFFECT OF THE LEVEL OF FAT IN THE DIET UPON UTILIZATION OF VITAMIN A [1]

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(Received for publication July 16, 1941)

This is a report on the second phase of an experiment planned to study the effect of various dietary factors upon utilization of vitamin A. Results reported in a previous paper (Muelder and Kelly, '41) indicated an effect of restricted intake of basal ration upon utilization of vitamin A. Because of the restriction in food intake, the calorie-control rats received not only fewer calories but less of all the nutrients (excepting the B vitamins) than did the positive control rats. It seemed probable that the restriction of some particular component of the basal ration may have been a causative factor in the consistently less favorable response to equal levels of vitamin A supplement for the rats on restricted food intake.

In view of the fact that vitamin A is a fat soluble vitamin, the role of fat in the basal diet as it affects the utilization of vitamin A seemed particularly pertinent. Possible effects on the occurrence of ophthalmia and on the depletion time, with the presence or absence of fat in the basal diet during depletion, have been observed by Nelson and Swanson ('32), Nakahara and Yokoyama ('28), and Culhane ('33); whereas Green ('34) and Lease and Steenbock ('39) observed that

[1] Approved by the director of the Michigan Agricultural Experiment Station for publication as Journal Article No. 465 (N. S.).

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widely varying percentages of fat in the basal diet did not appreciably affect survival time of rats on an A-free diet. These studies related to the depletion of vitamin A stores in the body. In the present investigation, body stores of vitamin A were depleted with the animals receiving a constant level of fat in the diet. The effect of the level of fat intake upon the utilization of graduated levels of vitamin A was then studied by means of diets containing different percentages of fat but adjusted to the same energy or calorie value by dilution with agar-agar. Rate of growth and incidence of "abscessed" areas were the criteria used to measure the response to graduated levels of vitamin A of 264 animals observed during the 2-year period, 1936 to 1938.

EXPERIMENTAL PROCEDURE

Triads of rats of the same sex and litter were started on the vitamin A deficient diet (table 1) at a weight of 50 to 55 gm. and an age of 25 to 28 days. At depletion, as determined by failure to gain in weight for 1 week and evidence of ophthalmia, the basal ration of the triads of rats was changed, each

TABLE 1

Composition of basal rations[1]

INGREDIENTS	DIET USED DURING DEPLETION	DIETS FOR EXPERIMENTAL PERIOD		
		Diet 0	Diet 5	Diet 10
Fat (Refined cottonseed oil): %	5	0	5	10
Casein[2]: %	18	18	18	18
Salt mixture[3]: %	4	4	4	4
Corn starch[4]: %	73	78	67	56
Agar-agar: %	0	0	6	12
Calories per 100 gm., calculated	409	384	385	386
% calories from fat	11.0	0	11.7	23.0

[1] Throughout the entire experiment 0.5 gm. bakers' yeast was fed daily as a supplement to supply the B vitamins.

[2] Crude casein purified for vitamin A assays according to Sherman, H. C., and Smith, S. L. The Vitamins, p. 257, 1931, second edition, Chemical Catalog Company.

[3] Osborne, T. B., and L. B. Mendel, J. Biol. Chem., vol. 32, pp. 309–323, 1917.

[4] Irradiated in a thin layer for 15 min. with a quartz mercury vapor lamp.

member of the triad receiving a different basal diet. The compositions of the three diets containing, respectively, 0, 5, or 10% fat and adjusted to the same caloric value through the addition of agar-agar, are shown in table 1. Thus, for the depletion period, each member of a triad received the same basal ration containing a constant level of fat, whereas during the experimental period, each member of the triad received a different basal diet, the three experimental diets being of the same caloric value per gram but differing in per cent of calories derived from fat. Each member of a triad, however, received the same level of vitamin A supplement, 0, 1, 3, or 6 International Units. Refined cottonseed oil was used throughout the experiment as the source of fat in the basal diet and as the diluent for the U. S. P. reference cod liver oil supplements (U. S. P., 1935). The same methods of preparation of reference cod liver oil dilutions and of administration of supplement employed in the previous work were used in this study. During the 6-week experimental period, the animals were weighed weekly, but the food consumption was checked daily. A quantitative uniformity in food consumption was insured by restriction of the food intake of two members of a triad to the level of the poorest feeder in that triad.

Gross autopsy findings were tabulated at the end of the experiment. As had been found in the previous study, a numerical total of the incidence of accumulations of keratinized epithelial cells, which were called "abscesses" seemed the most reliable and objective estimate of the utilization of the vitamin A.

DISCUSSION OF RESULTS

Male and female triads of rats were depleted on a diet containing 5% of fat in an average of 7.0 weeks. The growth plateau occurred at an averaged weight of 136.5 gm. Food intake averaged 58.6 gm. per week during the depletion period.

Effect upon growth

In table 2 are given summarized data for the 6-week experimental period for both sexes combined for the 2 years. Animals of the negative control group were usually found in a state of death rigor and an accurate measurement of length from tip of nose to tip of tail was not possible. Consequently, too few measurements of length of negative control animals were obtained to warrant presentation here. All of the average results were tested by the "t" test according to the formula $\text{"t"} = \frac{m - m_1}{\sqrt{\frac{(\gamma)^2}{n} + \frac{(\gamma_1)^2}{n_1}}}$ and the results are presented in table 3.

It will be noted from table 2 that, in general, with the negative groups, there was a tendency for a lower caloric intake and a greater loss of weight with a higher level of fat in the diet. There was also a shorter survival time on the higher levels of fat, the average length of survival time on the 0, 5, and 10% levels of fat being 16.1, 12.2, and 14.5 days, respectively. However, when these differences in average weight change during survival and number of "abscesses" at autopsy were tested by the "t" test, they were not found to be significantly different on different levels of fat (table 3, part 1). These results, then, regarding the relation of levels of fat in the diet to complete depletion of body stores of vitamin A are in agreement with findings of Green ('34) and Lease and Steenbock ('39).

Reasonably uniform caloric intakes were maintained within groups of animals at all levels of vitamin A administration. At the level of 1 I. U. of vitamin A, the groups of animals receiving 10% fat had an average weekly caloric intake which was 5.47 calories greater than that consumed by rats receiving no fat. However, it was expected that this increase in the calories consumed by rats receiving 10% fat would give only about 0.03 gm. gain in body weight per week, if calculated from the average gain per calorie ingested by these animals (0.005 g $\times$ 5.47). Actually, the 10% fat group showed a 2.5-gm. increase in weight per week over the 0% fat group on this

TABLE 2

Average weights, food consumptions, and autopsy findings on all triads during 6-week experimental period.

YEAR AND DESCRIPTION OF TRIADS	LEVEL OF VITAMIN A	ANIMAL	WEEKLY AVERAGE			GAIN PER CALORIE INGESTED	LENGTH RAT AT END OF EXPER.	"ABSCESSES" EVIDENT AT AUTOPSY [2]
			Food intake [1]	Calorie intake	Weight change [1]			
	units [3]	*no.*	*gm.*	*cal.*	*gm.*	*gm.*	*inches*	
1936–1938 Males and Females								
0% Fat	0	22	26.0 ± 1.54	99.84	−14.9 ± 1.41	−0.149		4.0 ± 0.30
5% Fat	0	22	23.3 ± 1.51	89.71	−15.2 ± 1.42	−0.169		4.1 ± 0.25
10% Fat	0	22	22.1 ± 1.81	84.31	−19.1 ± 1.48	−0.227		4.2 ± 0.29
1936–1938 Males and Females								
0% Fat	1	22	45.5 ± 0.91	176.72	−1.5 ± 0.71	−0.008	13.37 ± 0.15	1.1 ± 0.20
5% Fat	1	22	46.8 ± 0.87	180.18	+0.7 ± 0.72	+0.004	13.45 ± 0.16	1.1 ± 0.29
10% Fat	1	22	47.2 ± 0.76	182.19	+1.0 ± 0.63	+0.005	13.47 ± 0.11	1.1 ± 0.27
1936–1938 Males and Females								
0% Fat	3	22	56.4 ± 1.14	216.58	+3.6 ± 0.71	+0.017	13.68 ± 0.12	1.0 ± 0.29
5% Fat	3	22	57.3 ± 0.97	220.61	+5.2 ± 0.74	+0.024	13.88 ± 0.11	0.5 ± 0.14
10% Fat	3	22	57.2 ± 0.98	220.79	+6.2 ± 0.75	+0.028	13.85 ± 0.11	0.6 ± 0.21
1936–1938 Males and Females								
0% Fat	6	22	63.2 ± 1.21	242.69	+5.5 ± 0.76	+0.023	13.86 ± 0.15	0.6 ± 0.17
5% Fat	6	22	62.6 ± 1.20	241.01	+7.4 ± 0.81	+0.031	13.93 ± 0.14	0.8 ± 0.24
10% Fat	6	22	63.1 ± 1.16	243.57	+8.6 ± 0.75	+0.035	14.18 ± 0.17	0.7 ± 0.19

[1] Arithmetic mean ± standard error.

[2] Areas that most frequently showed "abscesses" were middle ear, base of tongue, lymph glands in neck, salivary glands, and genito-urinary tract.

[3] International Unit.

TABLE 3

Results of "t" tests for significant differences in average weight change, length, or number of "abscesses" in animals on different levels of vitamin A or fat.

GROUPS OF ANIMALS COMPARED 1.	AVERAGE WEIGHT CHANGES ON DIFFERENT LEVELS OF FAT			LENGTH OF ANIMAL ON DIFFERENT LEVELS OF FAT			NUMBER OF "ABSCESSES" ON DIFFERENT LEVELS OF FAT		
	0% & 5%	0% & 10%	5% & 10%	0% & 5%	0% & 10%	5% & 10%	0% & 5%	0% & 10%	5% & 10%
With no vitamin A	—	—	—				—	—	—
With 1 I. U. vitamin A	+	++	—	—	—	—	—	—	—
With 3 I. U. vitamin A	—	+	—	—	—	—	—	—	—
With 6 I. U. vitamin A	—	+	—	—	—	—	—	—	—

2.	AVERAGE WEIGHT CHANGES ON DIFFERENT SUPPLEMENTS OF VITAMIN A, IN I. U.						LENGTH OF ANIMAL ON DIFFERENT SUPPLEMENTS OF VITAMIN A, IN I. U.			NUMBER OF "ABSCESSES" ON DIFFERENT SUPPLEMENTS OF VITAMIN A, IN I. U.					
	0 and 1	0 and 3	0 and 6	1 and 3	1 and 6	3 and 6	1 and 3	1 and 6	3 and 6	0 and 1	0 and 3	0 and 6	1 and 3	1 and 6	3 and 6
With no fat	++	++	++	++	++	—	—	+	—	++	++	++	—	—	—
With 5% fat	++	++	++	++	++	?	+	+	—	++	++	++	—	—	—
With 10% fat	++	++	++	++	++	+	+	++	—	++	++	++	—	—	—

— No statistically significant difference (all values of "t" under 2.06).

\+ Statistically significant difference (values of "t" between 2.07 and 2.28).

++ Highly statistically significant different (values of "t" between 2.81 and 8.26).

increase of 5.47 calories. On referring to table 3, which gives the results of the "t" tests, it will be seen that this difference in weight change is highly significant. Likewise, the 2.2-gm. gain in weight of the rats on diets containing 5% fat over those receiving no fat is significant. The 0.3-gm. gain of the 10% fat group over the 5% fat group, however, is not significant. It would seem, then, that inclusion of either level of fat in this basal diet was a factor in determining weight response when 1 I. U. of vitamin A was fed.

It will be seen from the records on average weekly weight change that at all levels of vitamin administration, there was a superior gain in weight with 10% of fat in the basal diet over the other two groups, and also that the 5% level of fat permitted a weight response superior to that obtained on the basal diet with no fat in it. However, at the 3- and 6-unit levels of vitamin A, the only differences in response which were significant were those between the lowest and the highest levels of fat (table 3). At the 1-unit level of vitamin A, the difference in response between the 0% fat group and 5% fat group was also significant. When receiving 1 I. U. of vitamin A, the animals were more sensitive to changes in the intake of other dietary factors. In studying utilization of vitamin A by means of weight response, the effects of any other dietary essential in aiding vitamin A assimilation will be less pronounced apparently at each increase in the level of vitamin administered.

It is interesting to see that the gain-per-calorie-ingested also showed the same relative superiority for the basal diet containing increasing levels of fat. Basu ('37) reported that absorption of vitamin A from the intestine was dependent upon the presence of a suitable fat. Ahmad and Drummond ('31) have shown that carotene is more nearly completely absorbed when the basal diet contains 10% fat than when it is fat-free. Drummond, Bell and Palmer ('35), making observation on a human patient, found both carotene and vitamin A present in colloidal form and closely associated with the highly dispersed fat. It is likely that the presence of fat

in the diets used in this study made possible a more complete absorption of vitamin A, which in turn aided in better utilization of the calories ingested, as indicated by the slightly higher gains per calorie on the fat-containing basal rations.

As would be expected, increasing the level of vitamin A resulted in increased gains in weight, irrespective of level of fat in the basal diet, and the more highly significant differences in weight response occur between the wider ranges of vitamin intake. It is recognized that a part of this increased gain in weight with each increase of vitamin intake is due to differences in caloric intake. In a previous study (Muelder and Kelly, '41), it was demonstrated that the percentage of gain in weight from caloric intake increased with each elevation of vitamin intake, the average per cent of the gain in weight due to caloric intake at these respective levels being 59% at the 1-unit level, 64% at the 3-unit level, and 66% at the 6-unit level of vitamin intake.

It has been suggested (Orr and Richards, '34) that gain in weight is not synonymous with growth, and that animals continued to grow in length in spite of insufficient vitamin A in the diet. Measurements of the rats from the tip of the nose to the tip of the tail were made at the end of the experimental period of all animals on 1-, 3-, and 6-unit levels of vitamin intake. The mean lengths for each group are presented in table 2 and results of the "t" tests for significant differences in these lengths are recorded in table 3. In no case was there a significant difference in length due to a difference in percentage of fat in the basal diet, and only at the 10% level of fat was there a highly significant difference in length between the 1- and 6-unit levels of vitamin intake. Differences in length of rat at the different levels of vitamin intake were not as pronounced as the differences in weight gains. The fact that the differences in length of the rat between the 1- and 6-unit group on all three basal diets and also between the 1- and 3-unit groups on the basal diets containing 5 and 10% fat were significant would indicate that level of vitamin intake does affect growth in length.

Effect on incidence of "abscesses"

The summarized data on incidence of accumulations of keratinized epithelial cells are presented in table 2 as the average number of "abscesses" evident at autopsy. The levels of fat in the diets used in this experiment had no effect on protection against the development of areas of cell proliferations. The differences in response on the different basal diets were not statistically significant at any level of vitamin intake (part I, table 3).

As was found in the previous report, the administration of even 1 International Unit of vitamin A to groups of rats produced a highly significant reduction in number of "abscesses" over those observed for groups receiving no vitamin A. The administration of either 3 or 6 units showed a slight reduction in number of "abscesses" below those exhibited by groups of animals given 1 unit of vitamin A; but again these differences are not statistically significant, as shown by the "t" tests.

Although negative control groups demonstrated a slightly higher incidence of "abscesses" in this experiment than in the previous experiment (4.0, 4.1 and 4.2 as compared with 3.2, 3.5 and 3.7), the administration of 1, 3, or 6 International Units of vitamin A was equally as effective in reducing incidence of such areas as similar levels had been previously. Averages of 1.1, 0.7, and 0.7 "abscesses" for groups of animals on the 1-, 3-, and 6-unit levels of vitamin A in this experiment compare favorably with 1.5, 1.1, and 1.1 "abscesses" for groups on the same respective levels of vitamin A in the previous experiment.

SUMMARY

The influence of the level of fat in isocaloric basal diets upon the utilization of vitamin A has been studied by means of rate of growth and incidence of accumulations of keratinized cells or "abscesses" in carefully controlled triads of rats.

Inclusion of 10% of fat in the basal diet aided absorption of vitamin A sufficiently to produce statistically significant gains in weight over a basal diet containing no fat, but not over a

basal diet containing 5% of fat. For the levels of fat and units of vitamin A intake used in this experiment, unitage of vitamin intake was a more important factor in the production of highly significant gains in weight than level of dietary fat.

Growth in length was not affected by level of fat in the basal diet, but a significant increase in length was obtained by increased vitamin A intake.

The level of fat in the basal diet showed no statistically significant influence upon "abscesses," at the levels of fat and vitamin intake used in this experiment. Administration of 1, 3, or 6 International Units of vitamin A to groups of animals produced highly significant reductions in number of "abscesses" as compared to incidence of "abscesses" in groups receiving no vitamin A, although there were no statistically significant differences in number of such areas between these levels of vitamin A.

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THE RESPIRATORY QUOTIENT OF PROTEIN OF THE DALMATIAN DOG [1]

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The metabolism of the Dalmatian dog is characterized by a larger excretion of uric acid than that of other breeds (Benedict, '15–'16, '16; Wells, '18). It has been shown (Henry, Magee and Reid, '34) that if alanine is oxidized to uric acid, as in birds, the theoretical respiratory quotient is 0.667, but if alanine is oxidized until urea is the end product, then the respiratory quotient (R. Q.) is 0.83. These two facts led us to study the total metabolism of a Dalmatian dog with particular reference to the R. Q. and the relation of the excretion of uric acid to the R. Q., as well as the influence of ingestion of protein upon the R. Q.

EXPERIMENTAL

The respiratory exchange of an adult, female Dalmatian dog (aged 7 years and weighing 15 kg.) was measured by an open-circuit respiration chamber, and the outgoing air was analyzed on a Carpenter gas-analysis apparatus. The observations included the effects of several days of fasting and the effects of ingestion of different amounts of casein or raw beef (warmed to body temperature), either immediately before or several hours prior to the experiments. Differences in the time relationships with respect to ingestion of food were purposely

[1] A preliminary report of this study was presented at the 35th Annual Meeting of the American Society of Biological Chemists in Chicago, Illinois, on April 18, 1941. J. Biol. Chem., vol. 140, p. xxiii (1941).

made, to establish whether there was storage of either fat or sugar from protein with a subsequent combustion of the same, which would be indicated by the level of the R. Q. at some period of time following the ingestion of protein. Half-hour periods of measurement, continuing for 1½ to 3 hours, were made on from 12 to 16 days in two series of experiments 11 months apart. In the first series (1937) the dog was in early pregnancy, although this was not known when the study began. Her pregnancy terminated on August 14, 1937, when she gave birth to three puppies. At the start of the 1937 series the urine was collected only in 24-hour periods, but on June 28, 1937, and thereafter and also in 1938 the dog was catheterized immediately preceding and following the respiratory exchange measurements, and the urine was collected for the rest of the day either by catheterization or by voluntary voiding.

DISCUSSION OF RESULTS

The results of the observations on total metabolism, R. Q., and urinary nitrogen elimination are shown in table 1. The non-protein R.Q.'s were calculated in the conventional manner, and the table shows that, with the exception of the beginning of the fasting periods, they are for the most part below 0.71, the R. Q. of fat, and in general the higher the percentage of oxygen used to oxidize protein, the lower is the non-protein R. Q. This is particularly true of the last two values in the table.

The urine did not show any signs of acidosis by a qualitative test, and the percentage of ammonia in relation to the total nitrogen in the urine was not high enough in any case to indicate the presence of acidosis. Therefore it was assumed that the combustion of fat was normal in this dog. Calculations were then made as to what the R. Q. of protein would be if this were the case. For this calculation it was assumed that the factor for oxygen for protein combustion per gram of urinary nitrogen is correct. From the total oxygen consumption was deducted the oxygen used in protein combustion. The remainder was considered to be that for fat combustion.

TABLE 1

Respiratory exchange and urinary nitrogen elimination of a Dalmatian dog.

DATE	CONDITION	EXPERIMENT BEGAN	DURATION	O_2 PER HALF HOUR	URINARY NITROGEN PER HOUR	RESPIRATORY QUOTIENT		PER CENT OF O_2 USED TO OXIDIZE PROTEIN	RECALCULATED PROTEIN R. Q.
						Total	Non-protein		
1937			*hr.*	*l.*	*mg.*				
June 17	24 hrs. without food	10:50 A.M.	2	2.72	...	0.891	....	...	....
June 18	48 hrs. without food	10:00 A.M.	2½	2.72	...	0.714	....	...	....
June 21	48 hrs. without food	10:00 A.M.	2½	2.96	124 [1]	0.724	0.713	12.4	....
June 22	3 days without food	10:00 A.M.	2½	3.02	124	0.714	0.701	12.1	0.739
June 23	4 days without food	9:45 A.M.	2½	2.12	131	0.717	0.697	18.3	0.743
June 24	5 days without food	10:00 A.M.	2½	2.23	146	0.696	0.671	19.2	0.653
June 25	6 days without food	9:45 A.M.	2½	2.32	133	0.692	0.670	16.9	0.632
June 26	7 days without food	9:22 A.M.	2½	2.70	133 [1]	0.699	0.683	14.5	0.656
June 28	9 days without food	9:37 A.M.	1½	2.39	158	0.703	0.677	19.6	0.677
June 28	25 mins. after 30 gm. casein	12:02 P.M.	3	2.52	249	0.715	0.678	29.1	0.726
June 29	17 hrs. 40 mins. after 70 gm. casein	9:40 A.M.	1½	2.52	237	0.701	0.661	27.8	0.681
June 30	18 hrs. 45 mins. after 100 gm. casein	9:45 A.M.	1½	2.34	416	0.737	0.665	52.5	0.759
July 1	ca. 18 hrs. after 175 gm. casein	10:00 A.M.	1½	2.30	515	0.734	0.598	66.1	0.743
July 2	20 hrs. after 300 gm. beef	10:00 A.M.	1½	2.41	401	0.726	0.652	49.1	0.740
July 3	16 hrs. after 300 gm. beef								
	39½ hrs. without food	9:35 A.M.	1½	2.38	545	0.749	0.636	67.6	0.765
July 6	ca. 22 hrs. after 400 gm. beef	10:00 A.M.	1½	2.48	...	0.721	....	...	....
July 7	18 hrs. after 400 gm. beef								
1938	ca. 22 hrs. after 400 gm. beef	10:00 A.M.	1½	2.43	441	0.740	0.668	53.7	0.764
	17 hrs. after 400 gm. beef								
May 3	25½ hrs. without food	9:53 A.M.	2	2.68	204	0.799	0.797	22.5	....
May 4	13½ hrs. without food	9:22 A.M.	2	2.65	215	0.829	0.837	24.0	....
May 5	14¼ hrs. without food	9:14 A.M.	2	2.63	142	0.805	0.805	16.0	....
May 6	38½ hrs. without food	9:16 A.M.	2	2.49	143	0.704	0.684	16.9	0.682
May 7	62½ hrs. without food	9:29 A.M.	2	2.62	126	0.692	0.674	14.1	0.613
May 8	3 days 14½ hrs. without food	9:26 A.M.	2	2.46	125	0.689	0.669	15.0	0.605
May 9	4 days 14½ hrs. without food	9:25 A.M.	2	2.53	122	0.683	0.663	14.3	0.574
May 10	34 mins. after 350 gm. beef	9:00 A.M.	2½	2.88	305	0.682	0.626	31.3	0.638
May 10	4 hrs. after 350 gm. beef	12:30 P.M.	3	2.82	550	0.720	0.608	57.7	0.727
May 11	25 hrs. after food	9:35 A.M.	2	2.29	134	0.695	0.672	17.2	0.638
May 12	35 mins. after 500 gm. beef	9:14 A.M.	1½	3.25	410	0.713	0.659	37.3	0.717
May 12	3 hrs. 46 mins. after 500 gm. beef	12:25 P.M.	3	3.34	673	0.742	0.650	59.5	0.761
May 13	10 hrs. 49 mins. after 350 gm. beef	9.49 A.M.	2	2.56	468	0.697	0.572	54.0	0.688
May 14	10 hrs. 40 mins. after 500 gm. beef	9:25 A.M.	2	2.72	714	0.720	0.430	77.7	0.721

[1] Assumed.

Multiplication of this remainder by the R. Q. of fat (0.71) gave the carbon dioxide produced in the fat combustion. Subtraction of this from the total carbon-dioxide production gave that ascribable to protein combustion, and the ratio of this latter to the oxygen used in protein combustion gave the recalculated R. Q. of protein. The results are given in the last column of the table and show, in general, much more uniform quotients than are found in the column for the non-protein R. Q.'s.

Calculations of the effect upon the R. Q. of assumed errors in measurement of the respiratory exchange and of the urinary nitrogen did not show that these low non-protein quotients could be due to errors in measurement. The experiments were purposely spaced at varying times after the ingestion of the protein-containing foods, but as no experiment showed at any time quotients high enough to indicate a period of carbohydrate combustion, it is concluded that the low non-protein R. Q.'s cannot be the result of formation of sugar from protein. The excretion of uric acid was not high enough in any case to account for the lowering of the non-protein R. Q. Therefore the hypothesis is proposed that the R. Q. of protein of the Dalmatian dog, although varying with the condition of the animal with respect to fasting and ingestion of food, is lower in general than the usually accepted R. Q. of 0.81 for protein.

SUMMARY

The respiratory exchange of a Dalmatian dog was measured during fasting and after the ingestion of casein or meat in various amounts. The urine was collected by catheterization. The majority of the non-protein R. Q.'s were below 0.71, a fact that cannot be ascribed to formation of sugar from protein or to excretion of uric acid. As the urine showed no signs of acidosis, the protein R. Q.'s were calculated on the assumption that the fat metabolism was normal. They were all well below 0.81, and the hypothesis is advanced that the R. Q. of protein of the Dalmatian dog is lower than that used in general for protein.

The respiratory exchange measurements were made by Mr. Basil James and the urine analyses by Mr. Martin Stankard.

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VITAMIN A REQUIREMENTS IN THE RAT. THE RELATION OF VITAMIN A INTAKE TO GROWTH AND TO CONCENTRATION OF VITAMIN A IN THE BLOOD PLASMA, LIVER AND RETINA

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TWO FIGURES

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In 1939 Goss and Guilbert reported on the minimum vitamin A requirements for the rat. These investigators used as an indicator of vitamin A deficiency the appearance of cornified epithelial cells in smears obtained from the vagina and came to the conclusion that 18 to 22 international units (I. U.) of vitamin A per kilogram of body weight represented the daily minimum requirement. This level of intake not only prevented the occurrence of cornified cells in the vaginal smear, but also brought about fairly good growth and, to all outward appearances, the animal seemed normal in every way. However, at this unitage no vitamin A was detected in the liver, the chief reservoir of vitamin A in the body.

The present study was undertaken to obtain information on the optimal vitamin A requirements for the rat, i.e., that amount of vitamin A intake which will bring about the most favorable nutritional state. For the appraisal of the vitamin A status, we used the following criteria: (1) growth; (2) vitamin A blood level; (3) liver reserve; (4) vitamin A concentration in the retina. A previous report on the relation of vitamin A intake to blood level and to liver storage has appeared elsewhere (Lewis, Bodansky, Falk and McGuire, '41).

EXPERIMENTAL

One hundred and sixty-four albino rats, 3 to 4 weeks of age, obtained from the same colony and having been reared under identical conditions, were divided into groups and were fed the following supplements of vitamin A daily: 0, 1, 2, 10, 25, 50, 100 and 1000 I. U., respectively. The basal diet was devoid of vitamin A and had the following percentage composition: casein 18, salt mixture (Osborne and Mendel) 4, irradiated dry yeast 8, starch 65, hydrogenated cottonseed oil 5. The vitamin A supplement used for rats receiving 100 units or less daily was a reference cod liver oil obtained from the U. S. Pharmacopoeia Committee, whereas the animals receiving 1000 units daily were given a special preparation of vitamin A which was concentrated by the Hickman process ('37)[1].

The rats were weighed twice weekly and after they had received these vitamin A supplements for 6 weeks, they were sacrificed by exsanguination 24 hours after the last dose of the vitamin. The vitamin A contents of their bloods, livers and retinas were then determined.

Depletion experiments, which will be described later in the paper, were carried out on sixty-one additional rats.

Determination of vitamin A in the blood. The method employed was based on the Carr-Price reaction and, except for slight modification, was in accordance with the technique described by Kimble ('39). As the diet was devoid of carotene, this substance was not found in the blood, liver or retina. It was necessary to pool the blood of two or three rats in order to have a sufficient quantity of blood for a vitamin A determination.

Determination of vitamin A in the liver. To a sample of liver[2] weighing between 0.5 to 1 gm., 0.3 to 0.5 ml. of 60% KOH and 5 ml. of 95% alcohol were added and the mixture

[1] The vitamin A concentrate was obtained through the courtesy of the Winthrop Chemical Company and contained 200,000 international units per gram.

[2] At the lower vitamin A intakes the whole liver, or a considerable portion thereof, was used for analysis.

was placed in a water bath at 70° C. until the tissue disintegrated, which usually occurred within 15 to 20 minutes. The remainder of the procedure was the same as that employed in the determination of the vitamin A content of the blood.

Determination of vitamin A in the retina. The eyes were removed in bright daylight [3] immediately after the rats were sacrificed. A vertical section through the eyeball was made just distal to the lens, the anterior portion of the eye being discarded and the posterior portion being placed in a 4% alum solution for 5 minutes[4]. The retinas were then dissected from the remaining tissues and were placed in a previously weighed centrifuge tube containing 5–10 ml. of distilled water. The tube was shaken vigorously to free the retinas from any alum, and then centrifuged. The supernatant liquid was decanted after centrifugation and this procedure was repeated three times. After the last centrifugation, water adhering to the sides and the bottom of the tube, as well as to the packed retinas, was removed with small pledgets of filter paper until the weight of the retinas became constant. The retinas of at least six rats were required to carry out a vitamin A determination; the average weight of the retinas of six rats varied from 60 to 110 mg. depending upon the size of the rats.

Five milliliters of 95% alcohol and 0.1 ml. of 60% KOH solution were then added to the retinas and the mixture was placed in a water bath at 50–70° C. for 10 to 15 minutes, at the end of which time complete disintegration of the retinas usually resulted. The remainder of the procedure was the same as that used for the determination of vitamin A in the blood.

[3] The vitamin A content of the retina was found to be in the same range whether the eyes were removed in the dark from fully dark adapted animals or whether they were removed in bright daylight from rats who had been previously exposed to a bright light (100-watt lamp) for 5 minutes.

[4] The question may be raised whether alum causes any destruction of vitamin A in the retina. Experiments which we carried out revealed that the treatment of the retina with 4% alum causes no destruction of vitamin A and does not interfere with the determination of vitamin A in the procedure we employed.

RESULTS

The relation of vitamin A intake to growth. The rate of growth of the various groups of rats is shown in figure 1. It will be observed that the group receiving no vitamin A gained fairly well for the first 2 weeks, but after this period there was a slowing up in the rate of gain and from the fourth to the sixth week the weight remained stationary. Many of the rats in this group showed evidences of xerophthalmia after having received the basal diet for 6 weeks.

The rate of gain in weight among groups of rats receiving 1, 2, 10 and 25 units, respectively, was directly related to the

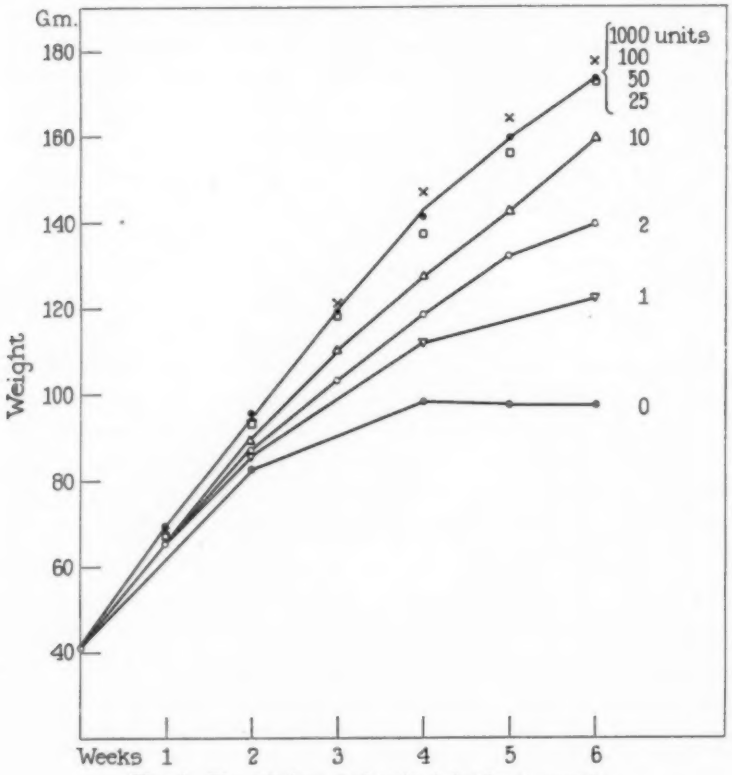

Fig. 1 The relation of vitamin A intake to growth.

intake. Thus, each increase in the amount of vitamin A fed resulted in a corresponding increase in the rate of gain in weight. On the other hand, increasing the intake above 25 units to levels of 50, 100 and 1000 units daily did not bring about a significant improvement in the weight curve.

Relation of vitamin A intake to blood level and to liver storage. The relation of vitamin A intake to vitamin A blood level and to liver storage is shown in table 1. When the intake was less than 50 units daily, the vitamin A concentration of

TABLE 1

Relation of vitamin A intake in the rat to vitamin A blood level, liver storage and vitamin A concentration in the retina.

VITAMIN A GIVEN DAILY FOR 6 WEEKS	ANIMALS IN GROUP	BODY WEIGHT		VITAMIN A		
		Initial (average)	At end of six weeks (average)	In blood plasma (average)	In liver (average)	In retina [1] (average)
I. U.		*gm.*	*gm.*	*I. U./100 ml.*	*I. U./gm.*	*I. U./gm.*
0	13	45	98	0	0	14
1	11	44	119	7	0	—
2	31	40	140	14	0	20
10	31	40	159	35	0	25
25	16	41	172	69	3	20
50	15	41	172	100	34	—
100	21	42	173	112	113	26
1000	26	43	177	110	1270	25

[1] The average concentration of vitamin A in the retina for the group receiving no supplement of vitamin A was computed from analyses carried out in forty-two rats, and for the groups receiving 2 units daily, retinal determinations were done in ninety rats.

the blood was directly related to the vitamin A intake. Increasing the feeding above 50 units daily had no appreciable effect on the vitamin A level in the blood, the average value being 112 units for rats receiving 100 units daily and 110 units for those receiving 1000 units daily. Thus, the minimum intake which brought about maximal blood levels was 50 units daily.

Storage of vitamin A in the liver was not found in any of the rats receiving 10 units or less daily. A small amount of vitamin A was detected in the livers of those receiving the 25 unit intake, an average of only 3 units per gram of liver

having been found. Increasing the intake above 25 units resulted in corresponding increases in the vitamin A storage of the liver. Thus, in contrast to the vitamin A content of the blood where maximal values were reached with an intake of 50 units, the liver concentration of vitamin A continued to mount as the daily intake was increased from 25 to 1000 units.

Relation of vitamin A intake to concentration in the retina. The vitamin A concentration in the retina is of particular interest because, as shown by Wald ('35), this vitamin plays an important role in the photochemical processes which take place in this organ during dark adaptation.

Vitamin A determinations of the retina were carried out on groups of rats receiving daily 0, 2, 10, 25, 100 and 1000 units respectively. The results of these analyses (table 1) revealed approximately the same values for rats receiving 2 or more units daily. On the other hand, rats receiving no vitamin A in their diets for a period of 6 weeks showed a diminished amount of vitamin A in their retinas.

It is of interest to analyze the retinal concentrations obtained within each group. Of seven determinations carried out in rats receiving no vitamin A supplements, five values were 15 units or lower. On the other hand, in the group of rats receiving 2 units daily, four of fifteen analyses revealed values below 15 units, and in the groups receiving 10, 25, 100 or 1000 units, one of fourteen analyses was below 15 units. Thus, in the majority of instances the 2-unit intake brought about maximal vitamin A concentrations in the retina.

Effect of enormous doses on vitamin A concentration in the blood, retina and liver. In view of the fact that the daily ingestion of as many as 1000 units of vitamin A did not bring about higher blood or retinal concentrations than did the feeding of considerably smaller amounts, the question arose as to whether it is possible to bring about abnormally high concentrations in the blood and retina by feeding enormous amounts of vitamin A. We therefore gave twenty rats 100,000 units of vitamin A daily for 4 weeks. The animals were sacrificed 48 hours after the last dose in order to be sure that the

blood concentration was at a post-absorptive level. Vitamin A determinations were then carried out on the bloods, retinas and livers. The results of this experiment revealed abnormally high concentrations of vitamin A in the blood and in the retina, the average blood level being 478 units per 100 cc. of plasma, and the average retinal content being 75 units per gram. The amount of vitamin A found in the liver was enormous, the average value being 19,200 units per gram. It should be mentioned that after receiving this high vitamin A intake for a few days, the animals began to lose weight, and in the course of 2 to 3 weeks became emaciated and lethargic, and several of the rats suffered a loss of hair; six died before the completion of the experiment.

The effect of vitamin A depletion on the concentration of vitamin A in the blood, retina and liver. In order to ascertain the relation of the amount of vitamin A stored in the liver to the maintenance of high blood and retinal levels during periods of depletion, three groups of rats were fed varying quantities of vitamin A and were then placed on a diet devoid of this vitamin. The first group, consisting of eighteen rats, was fed 100 units of vitamin A daily for 2 weeks; the second group, consisting of twenty-four animals, was given 100 units daily for 6 weeks and the third group of nineteen animals was fed 1000 units daily for 6 weeks. Prior to depletion, six animals from each group were sacrificed and the vitamin A content of their bloods, livers and retinas was determined.

After the vitamin A-free diet had been given for periods of 6 and of 9 weeks, six rats from each group were sacrificed and the vitamin A content of the livers, bloods and retinas was then determined. In addition, six animals in the second group were kept on a vitamin A-free diet for 20 weeks, at the end of which time similar analyses for vitamin A were carried out.

The results of this experiment are given in figure 2. It will be noted that, whereas there was a considerable drop in the blood levels in the first two groups of rats, in the third group,

which had an exceedingly large reserve of vitamin A in the liver, the blood level continued to remain high despite the administration of a vitamin A-free diet for 9 weeks. Apparently, the large stores of vitamin A in the livers of the third group maintained the bloods at optimal levels.

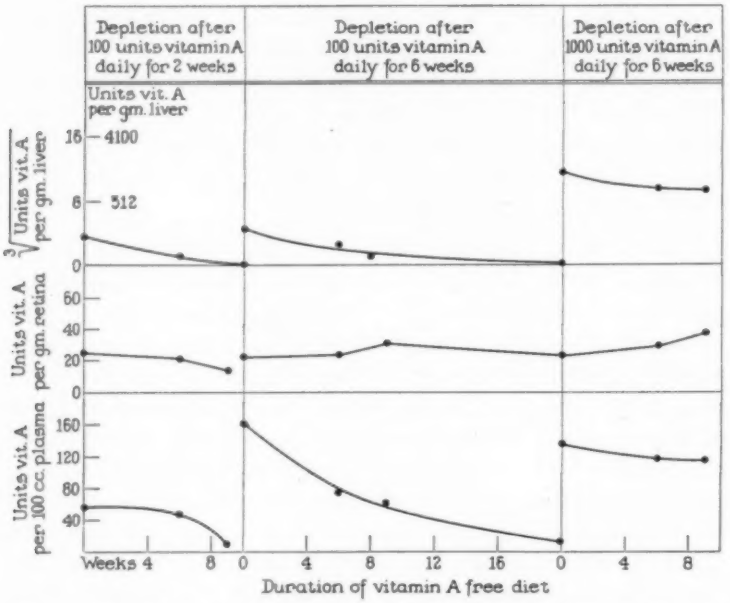

Fig. 2 The effect of a vitamin A-free diet on the concentration of vitamin A in the blood, in the liver and in the retina of rats which were previously fed varying amounts of vitamin A.

The retinal concentrations of vitamin A remained high in all three groups of rats throughout the greater part of the depletion period. In the first group of rats, which had a small storage of vitamin A at the beginning of the depletion period, the retinal concentration was observed to be low, 14 units per gram, only after the vitamin A-free diet had been given for 9 weeks, at which time most of the animals showed a loss of weight and were suffering from xerophthalmia. On the other

hand, in the second group of rats, which had a moderate reserve at the beginning of the depletion period, the retinal concentration remained high even after 20 weeks on a vitamin A-free diet, at which time the blood level was low and the liver was devoid of vitamin A. In this connection it should be added that Popper ('40) also found that during periods of depletion the retina contained vitamin A long after other tissues had been depleted. In Popper's investigation the organs were examined microscopically under ultra-violet light and the amount of vitamin A was estimated by the degree of green fluorescence.

DISCUSSION

Under the conditions of this experiment daily feedings of 2 units were required to prevent gross and histological evidences of vitamin A deficiency [5] and this intake may be regarded as the minimum vitamin A requirement. At this level of feeding, however, the blood concentration of vitamin A was markedly reduced and the liver contained no vitamin A reserve. As the 2-unit group received about 20 units of vitamin A per kilogram of body weight, the minimum requirement was the same as that previously reported by Goss and Guilbert ('39).

As previously stated, most of the rats whose daily intake was 2 units had maximal vitamin A concentrations in the retina. Although we did not carry out dark adaptation tests, it is probable that the animals in this group would have had normal vision in the dark. In this connection, it is of interest to mention that an intake of approximately 20 units per kilogram of body weight was found just sufficient to permit normal dark adaptation in cattle, swine and sheep (Guilbert, Miller and Hughes, '37), and in infants (Lewis and Haig, '39).

The amount of vitamin A which was required to bring about optimal growth in the rat was considerably higher than the

[5] We are indebted to Dr. Henry Brody of the Department of Pathology of Beth Israel Hospital for the histological examinations.

minimum requirement. For optimal gain in weight, a daily intake of 25 units was necessary. Since we did not employ levels of intake between 10 and 25 units daily, it is possible that amounts lower than 25, but greater than 10 units would also have resulted in optimal growth. Thus, the optimal intake, as judged by gain in weight, lies between five to twelve and one-half times the minimum requirement.

It may be recalled that the maximal blood concentrations averaged about 100 units per 100 ml. of blood plasma and were attained when the amount of vitamin A ingested daily was 50 units. Since we did not study the effect of levels of feeding between 25 and 50 units daily, it is possible that an intake of less than 50, but greater than 25, would also have yielded maximal blood concentrations. Thus, the smallest amount of intake required to bring about maximal levels of vitamin A in the blood is somewhere between twelve and one-half and twenty-five times the minimum requirement.

The question arises whether maximal blood values are also optimal, i.e., the most desirable, physiologically. The fact that, in the depletion experiments, rats having large quantities of vitamin A in their livers continued to maintain levels of about 100 units of vitamin A per 100 ml. of blood plasma for long periods of time when they were placed on a vitamin A-free diet, would indicate that the organism strives to maintain this level at the expense of the vitamin A reserve in the liver. It would therefore seem that concentrations of about 100 units per 100 ml. represent optimal blood levels.

It is difficult to determine what amount of vitamin A intake is required to bring about optimal concentrations in the liver inasmuch as we have no accurate way of determining what constitutes an optimal reserve. Certainly an intake of 25 units was too low, as storage in the liver was negligible at this level of feeding. An intake of 50 units daily provided a liver reserve (an average of 34 units per gram) which was of a magnitude that maintained the animal in a good state of nutrition for a period of 6 weeks of depletion, but at the end of 9 weeks, loss in weight and xerophthalmia developed. On the other hand,

rats receiving 100 units daily had a storage of vitamin A in their livers (an average of 113 units per gram) which was sufficient to keep them in a good nutritional state for a period of 20 weeks, during which time a vitamin A-free diet was given. Although we cannot state categorically what intake, under the conditions of our experiment, was optimal insofar as liver reserve is concerned, yet it would seem that an intake of 100 units daily (fifty times the minimum requirement) brought about a very satisfactory store of vitamin A in the liver.

The observations reported in these experiments are of special interest when compared with those noted in infants. Thus, Lewis and Haig ('39) found that the minimum vitamin A requirement in infants, as judged by dark adaptation, was of the same order as that obtained in rats, namely, 20 units per kilogram of body weight. At this intake the infant grew fairly well and appeared normal in every way. However, we have subsequently determined that at this level of intake the concentration of vitamin A in the blood is abnormally low (Bodansky, Lewis and Haig, '41). Optimal blood concentrations occurred in infants as in young rats, when the vitamin A intake was approximately twenty-five times the minimum requirement.

The maintenance of maximal concentrations of vitamin A in the retinas of the rats even when the vitamin A intake was so low as to bring about suboptimal growth, lowering of the vitamin A blood level and depletion of the vitamin A reserve in the liver is an extremely interesting finding. This observation would suggest that the ability of the eye to adapt to darkness (which is presumably dependent upon the vitamin A supply in the retina) is not affected early in the course of vitamin A deficiency. This view is contrary to general opinion, but recent investigations by Lewis, Bodansky and Haig ('41) lend support to this contention. For example, it was observed that several infants who had received a diet almost devoid of vitamin A for several weeks showed low blood concentrations of vitamin A, but dark adaptation remained

normal. Furthermore, twenty-two older children were found to have subnormal levels of vitamin A in the blood associated with normal dark adaptation. In view of these experiences we have come to the conclusion that the blood concentration is a more sensitive indicator of vitamin A deficiency than is the dark adaptation test.

SUMMARY

When vitamin A was fed daily at different levels to groups of 3 to 4 week old rats for a period of 6 weeks, the rate of growth was found to increase until it reached an optimum at an intake of 25 I. U. daily; it remained at this optimum for intakes up to 1000 units daily. The average plasma vitamin A concentration also increased with increasing levels of vitamin A intake, reaching an optimum at an intake of 50 units daily, and remained at this optimum for intakes up to 1000 units daily. There was no liver storage at intakes of 10 units or less daily, slight storage at 25 units, and increasingly larger storage at higher intakes. Retinal concentrations of vitamin A were usually low at zero units intake and reached an optimal value at an intake of 2 units daily. When rats were placed on a vitamin A-free diet, the plasma vitamin A concentration remained high in the animals with large liver stores, but fell rapidly in those with low reserves. The retinal concentration of vitamin A, however, remained high despite the low vitamin A plasma concentration and the absence of vitamin A in the liver. The minimum vitamin A requirement in the rat was 2 units daily or 20 units per kilogram. Optimal growth occurred at 25 units daily intake, optimal blood concentrations at 50 units daily, and good liver reserves at 100 units daily. Minimum and optimum vitamin A requirements are of the same order in the rat and infant.

We wish to express our appreciation to Mr. Bertrand Flusser, to Miss Mildred Weinstock and to Dr. Charles Haig for their cooperation in this study.

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THE EFFECTS OF THE SUBSTITUTION OF BICARBONATE FOR CHLORIDE IN THE DIET OF RATS ON GROWTH, ENERGY AND PROTEIN METABOLISM [1]

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This experiment was designed primarily to study the effects of a deficiency of dietary chloride in relation to the utilization of protein and energy, and serves incidentally to exhibit the effects of prolonged intake of bicarbonate as a prominent dietary constituent.

The published studies of the physiological functions of the chloride ion have been concerned, in the main, with its specific function in gastric juice, and with its role in maintaining the constancy of the internal environment of the tissue cells, with reference both to physiological neutrality and to the distribution of water and electrolytes. Chloride ions are considered as relatively passive participants in the maintenance of the ionic balance of the tissues (Irving and Manery, '36), especially because they do not enter into oxidation-reduction systems, do not form natural organic compounds, and have little specific influence on tissues.

In few investigations, therefore, has attention been given to the effects of a deficiency of dietary chloride on growth, on the utilization of protein, and on its relation to the utilization of dietary energy.

Osborne and Mendel ('18) and St. John ('28) observed that diets containing as little as 0.035 or 0.05% chlorine were

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quite adequate for growth. However, retardation of growth has been noted with more extreme limitations of dietary chloride by Orent-Keiles, Robinson and McCollum ('37) and Marquis ('38). Improvement in growth by the addition of sodium chloride to natural diets has been attributable to the sodium ion more than to the chloride ion (Mitchell and Carman '26; Miller, '26; St. John, '28).

The synthetic chloride-deficient ration used in the present investigation contained 0.02% chloride, and the control ration 0.28% chloride. The chloride in the salt mixture of the latter ration was replaced by equivalent bicarbonate in making up the chloride-deficient ration which, consequently, contained 0.49% bicarbonate. In this quantity, bicarbonate seems to be harmless (Olson and St. John, '25; Kahlenberg, Black and Forbes, '37) and the results obtained with the low-chloride diet are considered to represent mainly the effects of the chloride deficiency.

EXPERIMENTAL

A comparison was made of a chloride deficient diet with one of normal chloride content by means of a metabolism and body analysis experiment using as subjects twelve litter-mate pairs (seven males, five females) of albino rats from a colony originating in the Wistar strain.

The basic experimental diet was of the following percentage composition: sucrose 65, casein 20, hydrogenated vegetable oil 10 [2], cellu flour 2, and mineral salt mixture 3. The salt mixture was basically that of Hubbell, Mendel and Wakeman ('37), the sodium and potassium chlorides of this mixture being replaced by equivalent quantities of bicarbonates of the same cation in the chloride deficient ration.

In addition, each 100 gm. of the ration contained the following vitamin supplements and accessory food factors: carotene 3 mg., viosterol [3] 100 mg. (333 units of vitamin D), thiamine

[2] Crisco.

[3] Squibb, irradiated ergosterol in oil 250 D.

hydrochloride 0.5 mg., riboflavin 0.5 mg., pyridoxine 0.5 mg., calcium pantothenate [4] 0.25 mg. and lecithin [5] 0.15 gm.

By analysis the ration contained 98.6% dry matter, 2.89% nitrogen and 4.66 Calories per gram.

Both rats of a pair received the same amount of the equicaloric diets, the quantity fed being determined daily for each pair by that eaten on the preceding day by the individual which consumed the lesser amount.

The details of experimental technic were essentially as described by Swift, Kahlenberg, Voris and Forbes ('34).

The rats were allowed distilled water ad libitum and the water consumption was determined by weighing the water bottles at appropriate intervals.

Urine and feces were collected daily, and at the end of 10 weeks the paired rats were killed and the bodies analyzed for water, nitrogen, fat and energy.

The total 10-week collections of urine and feces were analyzed for nitrogen and energy. The nitrogen aliquot of the urine was preserved with sulfuric acid and the energy aliquot was dried without preservation. The energy of the dried sample was corrected for the loss of nitrogen in drying on the assumption that it was lost as urea.

Twelve rats (six male, six female) corresponding in age, weight and heritage to the paired rats, were killed and analyzed for alimentary fill, water, nitrogen, fat and energy to represent the body composition of the experimental rats at the start of the experiment. Average analyses for males and females were used separately.

Also, ten rats (seven male, three female) were fed the normal chloride ration ad libitum in order to determine its adequacy for growth.

For the sake of brevity, in discussing the results, the rats which received the chloride-deficient experimental diet are designated the bicarbonate rats, and those which received the diet of normal chloride content are referred to as the chloride rats.

[4] Donated by Merck and Co.

[5] Pfansteil Chemical Co. Pure lecithin.

RESULTS

The rats partook well of the experimental rations and there was no overt evidence of vitamin deficiency.

The rats fed ad libitum on the normal chloride diet thrived and appeared healthy throughout the experiment. The male rats in this group made an average weekly gain of 15.9 gm. on an average food intake of 72.2 gm. per week for the 10 weeks. The female rats made an average gain of 12.6 gm. per week with a food intake of 64.3 gm.

In the experimental group the food intake of the paired rats was invariably limited by the bicarbonate rats. The food intake for the 10 weeks was 531 gm. for the male rat pairs and 508 gm. for the females (table 1). Since the bicarbonate rats

TABLE 1

The effects of the substitution of bicarbonate for chloride in the diet on the growth of rats and utilization of nitrogen over a period of 10 weeks.

	MALES		FEMALES	
	HCO_3^-	Cl^-	HCO_3^-	Cl^-
Feed intake, grams	531.0	531.0	508.0	508.0
Water intake, grams	864.0	705.0	1045.0	699.0
Final body weight [1], grams	147.6	176.7	129.1	158.5
Initial body weight [1], grams	63.8	63.7	53.2	54.0
Total body weight gained, grams	83.8	113.0	75.9	104.5
Fat gained, grams	7.0	12.3	9.5	15.4
Per cent of total gain	8.4	10.9	12.5	14.7
Fat-free tissue gained, grams	76.8	100.7	66.4	89.1
Water gained, grams	51.8	69.9	45.0	61.8
Per cent of fat-free tissue	67.4	69.4	67.8	69.4
Protein gained, grams	19.5	25.0	16.4	21.0
Per cent of fat-free tissue	25.4	24.8	24.7	23.6
Residual tissue [2] gained, grams	5.5	5.8	5.0	6.3
Per cent of fat-free tissue	10.6	5.8	7.5	7.1
Nitrogen intake, grams	15.36	15.36	14.68	14.70
Fecal nitrogen, grams	0.81	0.87	0.80	0.82
Nitrogen digested, grams	14.55	14.49	13.88	13.88
Per cent of intake	94.7	94.3	94.6	94.4
Urinary nitrogen, grams	10.76	9.99	10.73	9.98
Nitrogen balance, grams	3.79	4.50	3.15	3.90
Per cent of intake	24.6	29.3	21.4	26.5

[1] Does not include contents of alimentary tract.

[2] Residual tissue = non-protein, non-fatty, dry substance.

were fed essentially ad libitum, the food intake of the pair-fed chloride rats, compared with those fed ad libitum, was reduced by 26% for the males and 21% for the females.

The water consumption (table 1) was significantly greater for the bicarbonate rats than for the chloride rats. The difference was more marked for the females than for the males, but no explanation is offered for this observation. In one pair of male rats the water consumption was greater for the chloride rat while in two other pairs the amount of water drunk by the chloride and bicarbonate rats was practically the same. There were no such exceptions among the female pairs.

The growth differences and other data were remarkably consistent and, consequently, the average values reported in the tables are quite representative.

Growth

The growth data are presented in table 1. The total weight gained by the bicarbonate rats was, as an average, 73.4% of the total weight gained by their pair-mates on the normal chloride diet. From the analysis of the gains, the bicarbonate rats gained 73.5% as much water, 78% as much protein, 60% as much fat and 87% as much residual (mainly mineral) substance as the chloride rats.

On the basis of the total gain, there was no evident difference in the percentage of gain as water by the bicarbonate and chloride rats, but on the basis of the fat-free gain the percentage of the gain as water by the chloride rats (69.4 ± 0.5 [6]) was significantly greater than the percentage gain as water by the bicarbonate rats (67.1 ± 0.6), and the individual data were characterized by a high degree of consistency.

The percentage gain of protein was significantly greater for the bicarbonate rats than for the chloride rats on the basis of either total weight or the fat-free tissue gained. On the latter basis, protein represented 25.1% of the fat-free tissue gained by the bicarbonate rats and 24.2% of that gained by

[6] Standard deviation.

the chloride rats. Statistically, the odds were 370 to 1 against this difference occurring by chance alone.

The relationship of water and protein has a definite significance with respect to tissue cell structure. From the data of Forbes, Voris, Bratzler and Wainio ('38) the same ratio of water to protein gained prevailed for diets containing 25, 30, 35 or 45% protein. This value was 2.93 gm. of water gained for each gram of protein gained. In the present investigation the ratio of water gained to protein gained was 2.87 ± 0.11 [1] for the chloride rats and 2.70 ± 0.08 for the bicarbonate rats. Statistically the odds are greater than 10,000 to 1 that this difference is significant. Thus, there is no doubt that the tissue cells of the bicarbonate rats were water-poor because of the insufficiency of chloride in the diet.

Fat gained by the chloride rats represented a significantly larger proportion of the total gain than the fat gained by the bicarbonate rats and, conversely, the chloride rats gained a smaller proportion of residual substance.

Utilization of protein

The effects of dietary chloride deficiency on the disposal of dietary nitrogen are presented in table 1. The fecal nitrogen of the bicarbonate rats was significantly lower than that of the chloride rats, but this difference when related to the much greater nitrogen intake appeared to be negligibly small. The difference in the digestibility of nitrogen, therefore, cannot be emphasized.

The urinary nitrogen excretion was about 8% greater in the bicarbonate rats than in the chloride rats and, consequently, the latter showed a significantly higher nitrogen retention than the former. The nitrogen retention for the bicarbonate rats was 82.6% of that for the chloride rats. The corresponding value determined from the body analysis for nitrogen gained was 77.7%. These balances differ slightly in significance since the value determined by the collection of the excreta involves a small error on account of the contamina-

[1] Standard deviation.

tion of the feces with shed hair and scurf, while the balance determined by body analysis involves the error of failure to account for the nitrogen of the shed hair and scurf as having been utilized.

Protein gained (table 1) was computed from the body analysis data for nitrogen gained by using the factor 6.0 for converting nitrogen to protein. The significance of the differences in protein storage has been discussed in connection with the growth data.

Utilization of energy

The results of chloride deficiency as affecting the utilization of energy are presented in table 2. Corresponding to the slightly greater digestibility of nitrogen, the bicarbonate rats, likewise, digested a slightly greater percentage of their energy intake than did the chloride rats. In relation to metabolizable

TABLE 2

The effects of the substitution of bicarbonate for chloride in the diet of rats on the metabolism of energy over a period of 10 weeks.

	MALES		FEMALES	
	HCO_3^-	Cl^-	HCO_3^-	Cl^-
Energy intake, Calories	2477	2477	2368	2371
Fecal energy, Calories	138	168	139	144
Energy digested, Calories	2339	2309	2229	2227
Per cent of intake	94.4	93.2	94.1	93.9
Urinary energy, Calories	76	68	73	67
Non-metabolizable energy of body protein, Calories [1]	24	31	20	26
Metabolizable energy, Calories	2239	2210	2136	2134
Per cent of intake	90.4	89.2	90.2	90.0
Energy of body gain, Calories	180	259	186	269
Per cent of metabolizable energy	8.0	11.7	8.7	12.6
Energy gain as fat, Calories	66	116	90	146
Per cent of total gain	36.7	44.8	48.4	54.3
Energy gain as protein, Calories	114	143	96	123
Per cent of total gain	63.3	55.2	51.6	45.7
Heat production, Calories	2083	1982	1970	1891
Per cent of metabolizable energy	93.0	89.7	92.2	88.6

[1] Grams of nitrogen gained (body analysis) × 7.45 = Calories of non-metabolizable energy of body protein gained.

energy this difference was compensated, in part, by the higher urinary energy of the bicarbonate rats, but when the non-metabolizable fraction of the body protein stored was considered, the metabolizable energy was 90.2% of the energy intake compared with 89.6% for the chloride rats. Statistically, the odds are 200 to 1 against the difference occurring by chance alone.

However, the chloride rats stored 12.2% of their metabolizable energy as body gain, compared with 8.4% gained by the bicarbonate rats. Conversely, the chloride rats lost 89.2% of their metabolizable energy as heat, whereas the bicarbonate rats lost 92.6% as heat. Thus, the difference in heat production is accounted for by the inability of the bicarbonate rats to retain as large a proportion of their metabolizable energy as did the chloride rats.

The chloride rats exceeded the bicarbonate rats in energy gained both as protein and as fat, in absolute quantities. The percentage of the total energy gain as protein was smaller for the chloride rats than for the bicarbonate rats and, conversely, the percentage of the total energy gain as fat was larger.

DISCUSSION

In summarizing the comparison between the bicarbonate and chloride rats, the following effects may be enumerated as the result of a deficiency of dietary chloride: (1) depression of appetite, (2) increased consumption of water, (3) retarded growth, (4) smaller proportionate gain of fat and water, (5) larger proportionate gain of protein and residual (mainly inorganic) substance, (6) slightly decreased fecal nitrogen, increased urinary nitrogen and decreased nitrogen retention, (7) slightly greater metabolizability of energy, but a lesser gain of body energy and a higher heat production.

Thus, the nutritional effects of a deficiency of dietary chloride are, in many respects, quite similar to the effects of deficiencies of sodium and potassium (Orent-Keiles, Robinson and McCollum, '37; Kahlenberg, Black and Forbes, '37;

Orent-Keiles and McCollum, '41). In fact, the observed effects of chloride deficiency are not materially different from those of a general mineral deficiency, as described by Brooke and Smith ('33), Eppright and Smith ('37), and Kriss and Smith ('37, '38 a, '38 b). The workers last mentioned found that both the basal and the total energy metabolism were considerably greater with rats subsisting on mineral deficient diets than with rats receiving normal control diets. The increased total metabolism was accounted for largely by the increased basal metabolism. The mineral deficient rats oxidized relatively larger proportions of fat and smaller proportions of carbohydrate, while no difference was noted in the protein metabolized.

SUMMARY

In a paired-feeding experiment comparative data on water consumption, growth, energy and protein metabolism, and body composition were secured with rats on a synthetic diet with normal chloride content (0.28%) and rats on the same diet in which the chlorides of the salt mixture were replaced by equivalent quantities of the corresponding bicarbonates. The chloride deficient ration contained 0.02% chloride and 0.49% bicarbonate. At this level of intake, the bicarbonate was considered to be innocuous and the results obtained were attributed to the deficiency of dietary chloride.

In comparison with the rats receiving the normal chloride ration, the chloride deficient rats showed depression of appetite, increased consumption of water, increased heat production and diminished body gain of nitrogen and energy.

There was a smaller percentage gain of water on the basis of fat-free tissue, and the ratio of water gained to protein gained was significantly lower with the chloride deficient rats than with the normal controls.

The prevailing deficiency of chloride ion did not affect the digestion and absorption of nutrient energy but did prominently affect its disposal within the bodies of the rats.

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THE INTERRELATION OF CALCIUM AND FAT UTILIZATION IN THE GROWING ALBINO RAT [1]

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Several recent publications have indicated that the addition of fat to a fat-free diet has a favorable influence on calcium utilization, but variations in experimental conditions and dietary regimes have led to differences in conclusions.

Among the first to investigate this relationship were Holt, Courtney and Fales ('20), who attributed certain instances of poor calcium retention to the formation of insoluble calcium soaps from excess fatty acid in the intestine. Zucker and Barnett ('23) suggested that part of the antirachitic property of natural fats was due to the union of calcium with the fatty acids to form soaps, thereby conserving phosphorus which would otherwise have been excreted as insoluble calcium phosphate. Telfer ('26) noted that the faulty utilization of bone-forming elements in rickets seemed to be due to defective absorption. Westerlund ('34 a, '34 b, '40) fed the pure fats tripalmitin, triolein, tributyrin, and tristearin to adult rats. He noted a deleterious effect of tristearin and tripalmitin on calcium absorption, which was exerted directly in proportion to the molecular weights.

Knudson and Floody ('40) and Jones ('40) found that moderate levels of fat in the diet favored the utilization of calcium.

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The purpose of the present experiment is to obtain further information concerning the physiological relationship between fat and calcium in the rapidly growing animal.

EXPERIMENTAL PROCEDURE

The experimental subjects were twenty-eight normal male albino rats of The Wistar Institute strain. They were selected as quadruplets, four from a litter, 3 days after weaning, at which time the animals were 26 days old and weighed from 48 to 56 gm. Six sets of four litter mates each were selected initially, but due to the abrupt death of one individual, an additional set of four litter mates was chosen in which the rats were 29 days old and averaged 71 gm. in weight. Each rat of a set of litter mates was fed one of the four experimental rations.

The animals were on experiment for a period of approximately 6 weeks, which included a 4-day preliminary period and a 40-day period of excreta collection. The rats were kept in individual metabolism cages designed by Swift et al. ('34) which permitted the separate collection of the feces and the urine. Distilled water was available to the rats at all times.

At the end of the collection period the rats were sacrificed, the gastrointestinal tracts removed, and the acidity of the contents determined with the glass electrode Beckman pH meter.

Calcium was determined by the volumetric method of the Association of Official Agricultural Chemists ('40) in which the calcium is precipitated as the oxalate and titrated with permanganate. Ether extract was determined by the official, direct method of the Association of Official Agricultural Chemists ('40).

Preparation of rations

Four synthetic rations (table 1) were prepared from purified foodstuffs in such manner as to provide, when fed in equicaloric amounts, essentially the same contents of protein, salt

mixture, calcium, phosphorus, and vitamin supplements. The rations, however, contained 5, 15, 28 and 45% fat, respectively. Salts were provided in the Osborne and Mendel XXX mixture, as modified by Wesson ('32), and as further modified by eliminating its contents of calcium and phosphorus.

Calcium was included in the rations in the form of tricalcium phosphate, and additional phosphorus to furnish a calcium-phosphorus ratio of 1:1 was added in the form of dibasic potassium phosphate. Enough calcium was added to each ration so that a daily food intake of 30 Calories furnished 25 mg. of calcium. This level was chosen as being safely

TABLE 1

Composition of rations.

COMPONENTS	RATION			
	1	2	3	4
	%	%	%	%
Dextrin	70.82	58.27	39.89	16.08
Casein	18.88	21.28	24.82	29.39
Oleo oil	4.72	14.04	27.74	45.46
Celluflour	1.89	2.13	2.48	2.94
Salt mix	2.83	3.19	3.72	4.41
$Ca_3(PO_4)_2$	0.86	1.00	1.17	1.40
$K_2H\ PO_4$	....	0.09	0.18	0.32
	100.00	100.00	100.00	100.00

below the optimum, which is approximately 30 to 50 mg. per day in the young growing rat, and at the same time not low enough to cause a body demand strong enough to mask any effect of the fat on the calcium utilization.

The fat used in preparing the rations was a high-grade oleo oil, with a melting point of about 41.5°C. This fat was chosen because of its rather slow absorption rate, as compared with other fats (Steenbock, Irwin and Weber, '36). It was thought that a slow rate of absorption might conceivably enhance any effect which a fat might have on the utilization of calcium.

Vitamin-free casein was included in all four rations in excess of 19%, and provided equal amounts of protein in equicaloric portions of each of the four diets.

A purified dextrin was used as the carbohydrate. Dextrin was selected in preference to other carbohydrates because it has not been found to exert any influence on calcium retention.

The vitamin supplement, which was the same in equicaloric amounts of all four rations, supplied a daily average of 20 μg. beta-carotene, 25 I. U. irradiated ergosterol, 35 μg. thiamine hydrochloride, 35 μg. riboflavin, 35 μg. B_6 hydrochloride, 20 μg. calcium pantothenate, and 11 mg. of lecithin, which was included for its content of choline.

Determination of intestinal acidity

In order to determine the acidity of the intestinal contents that existed during the experimental period several precautions were taken. A time interval of 5½ hours was allowed to elapse between feeding and killing each rat, and quadruplets were fed at a specific hour for 3 days previous to the determination.

After a rat was killed in the gas chamber at the designated time, the entire gastrointestinal tract was carefully removed and placed between two layers of wet gauze to keep it moist. The duodenum was then removed and its contents expelled into the 0.5 ml. beaker of the Beckman pH meter. Three drops of distilled water were added and mixed thoroughly with the sample before inserting the electrodes and determining the pH value. This value was then checked by remixing and redetermining. The glass electrode was used for all pH determinations. A 4-inch section of the jejunum (6 inches below the duodenum) was removed next, and the contents treated similarly. The third determination was made on the contents of a 4-inch section of the ileum just above the cecum. The acidity of the stomach and cecum was determined last.

The slight dilution of each sample was necessary to increase the volume so that it covered the bulb of the glass

electrode when the 0.5 ml. beaker was used. Such a dilution did not affect the hydrogen ion concentration.

DISCUSSION OF RESULTS

The original plan of the experiment was to feed different diets in equicaloric amounts to each rat in a set of four litter mates, but after 10 days it became apparent that the high-fat ration was severely limiting the consumption of the other rations. Hence it was decided to feed the high-fat diet ad libitum, and to feed the other three diets as planned.

The rats which received equicaloric amounts of the 5, 15 and 28% fat rations grew rapidly at equal growth rates, but the rats receiving the smaller caloric intake of the 45% fat diet ad libitum grew at a slower rate. This was particularly true of rat no. 20 which weighed 52 gm. at the start of the experiment, reached a maximum weight of 61 gm. after 10 days, and then remained nearly constant in weight for the remainder of the 40-day collection period. During the collection period it consumed 17 Calories of the 45% fat ration per day. After the collection was completed, rat no. 20 was fed 17 Calories of the 5% fat diet daily, and proceeded to gain weight at the rate of 1 gm. per day during a 12-day test period.

A consideration of this trial leads to the conclusion that the high fat content of the 45% fat diet exerted a definite depressive effect on the growth rate as well as on the food consumption. It was noted that the rats on this diet excreted a larger number of fecal pellets than did the rats on the other three diets.

The average values for calcium utilization in per cent, summarized in table 2, from the 5, 15, 28 and 45% fat diets, were 80.0, 77.7, 74.1 and 46.8, respectively.

The statistical significance of these values was calculated by Love's modification of "Student's" method ('24). The calculated odds that significant differences exist between the average percentage values for calcium utilization are as follows: 5 and 15% fat, 5:1; 5 and 28% fat, 76:1; and 5 and 45% fat, 5000:1.

TABLE 2

Average calcium utilization and digestibility of fat during 40 days. Six animals on each ration.

RATION	CALCIUM					FAT			
	Intake	Excretion		Retention	Utilization	Intake	Excretion	Digested	
		Urine	Feces						
	mg.	*mg.*	*mg.*	*mg.*	*%*	*gm.*	*gm.*	*gm.*	*%*
1	765	16	137	612	80.00	12.20	1.33	10.87	89.10
2	739	15	150	574	77.67	30.20	1.12	29.08	96.29
3	761	18	179	564	74.11	50.18	0.96	49.22	98.09
4	709	17	360	332	46.83	59.88	1.30	58.58	97.83

It is apparent that the utilization of calcium decreased moderately and consistently in the order of the increase in the fat content of the diets from 5% to 28%, and that the utilization from the 45% fat diet was considerably less efficient. These results compare favorably with the work of Knudson and Floody ('40) who noted a similar superiority of 5% as compared with 10% or 20% of fat in the diet in the healing of rickets under the influence of vitamin D.

A study of the intestinal acidity, summarized in table 3, shows that a more acid condition in the intestine resulted from the 5% fat diet than from the diets richer in fat. The average pH values of the contents of the jejunum, in which the greater part of the calcium absorption takes place, were 5.81, 6.02, 6.18 and 6.65, for the 5, 15, 28 and 45% fat diets, respectively. The calculated odds that these values are statistically significant are as follows: 5 and 15% fat, 6:1; 5 and 28% fat, 40:1; and 5 and 45% fat, 434:1.

TABLE 3

Average pH values for intestinal acidity, expressed after 40 days on various fat intakes.

RATION	NO. OF ANIMALS	STOMACH	DUODENUM	JEJUNUM	ILEUM	CECUM
1	7	3.52	5.74	5.81	7.10	6.59
2	7	4.03	5.72	6.02	7.17	6.66
3	5	3.49	5.61	6.18	7.24	6.76
4	6	3.90	6.17	6.65	7.31	6.95

The utilization of calcium paralleled the acidity of the intestinal tract, the most efficient calcium utilization accompanying the most acid reaction. This lends emphasis to the theory of Boyd, Crum and Lyman ('32) that a moderate amount of fat in the diet favors the absorption of calcium by maintaining a favorable acidity in the intestine. This theory does not explain, however, the increasingly poorer utilization of calcium, and the accompanying decrease in acidity, in the order of the increase in fat from 5% to 15, 28 and 45%.

The most efficient calcium utilization was obtained when the diet contained 1 gm. of fat to 0.063 gm. of calcium, and the utilization of calcium decreased as the fat-calcium ratio increased. These results agree with the work of Hickmans ('24) who found that the absorption of calcium by children was most efficient when the food contained 1 gm. of fat to 0.04–0.08 gm. of calcium.

Klinke ('28) studied the solubility of calcium-fatty acid-bile complexes and noted that calcium soaps formed very soluble compounds with desoxycholic acid. He also found that excluding the bile from the intestines of dogs by means of bile fistulae greatly increased the amount of calcium in the feces. Beznák ('31) continued this work and found that the addition of sodium taurocholate to a diet containing calcium and fat materially increased the absorption of calcium. He stated that bile salts play an important role in calcium absorption.

Verzár ('36) suggested that through the hydrotropic action of bile, bile acids form complexes with the calcium and fatty acids that are soluble in water and that can diffuse through the intestinal wall. He also proposed a similar theory for the absorption of fat: the fatty acids, through the hydrotropic action of the bile, form a bile-fatty acid complex (1 molecule of fatty acid to 3 of bile acid) that is water-soluble and readily absorbable by the intestine.

This hydrotropic action theory has been widely accepted to explain the passage of fat through the intestinal epithelium, and it seems possible that part of the calcium might be ab-

sorbed in a similar manner. The work of Irving ('26) shows that water-soluble, ionized calcium can be absorbed without bile in an acid medium, which would serve to explain the absorption of some calcium that takes place on a fat-free, or on a high-fat diet.

If it is assumed that fat affects calcium absorption through the formation of bile-fatty acid-calcium complexes, a necessary condition would be that enough bile be present both for the formation of these complexes and for the absorption of the major part of the fat. An inspection of table 2 suggests that enough bile was present to produce a nearly complete digestibility of the fat in the high-fat diet, but it is possible that nearly all of the bile was utilized for that purpose and that a minimum remained for the formation of bile-fatty acid-calcium complexes.

The increased alkalinity of the intestine after the feeding of the high-fat diet may be due partly to the removal of the bile acids and fatty acids during absorption of the fat, and partly to an increase in the flow of the bile and pancreatic secretions which are alkaline. This flow is stimulated by the ingestion of fat.

It seems probable from the work of Jones ('40) that vitamin D is necessary in the formation or absorption of such bile-fatty acid-calcium complexes in the intestine. In the absence of this vitamin Jones obtained no increase in calcification from feeding sodium oleate, or the calcium soaps of lard, while Boyd, Crum and Lyman ('32) found that the reverse was true when vitamin D was included in the diet.

The inferior calcium retention that resulted from fat-free diets, reported by several investigators, could be explained on the basis that no fat was present for the formation of bile-fatty-acid-calcium complexes. The decreased acidity noted by Boyd, Crum and Lyman ('32), under such conditions, also seems to be a factor contributing directly to a lowered calcium absorption.

The results of experiments now in progress, using similar techniques to determine the percentage fat intake for optimal calcium retention, will be reported later.

SUMMARY

Excellent growth was obtained from diets containing 5, 15 and 28% fat, but 45% of fat in the diet resulted in less growth and in the excretion of a larger number of fecal pellets.

The utilization of calcium decreased moderately and consistently in the order of the increasing fat content of the diets from 5 to 28%, and then decreased considerably for the 45% fat diet.

A more acid condition in the intestine resulted from the 5% fat diet than from the diets richer in fat. The utilization of calcium paralleled the acidity of the intestinal tract, the most efficient utilization accompanying the most acid reaction.

The most efficient calcium utilization was obtained from the diet containing 1 gm. of fat to 0.06 gm. of calcium; and the efficiency of the utilization of calcium decreased in the order of the increase in the ratio of fat to calcium in the diets.

The data are discussed in relation to various theories advanced to explain the physiological relationship between fat and calcium; and it is suggested that at least two factors are involved: (1) the acidity of the intestinal tract, and (2) the formation of readily absorbable bile-fatty acid-calcium complexes.

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THE INFLUENCE OF DIETARY FAT ON LACTATION PERFORMANCE IN RATS

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A dozen years ago Maynard and McCay ('29) reported that the removal of most of the lipids by solvent-extraction from a grain mixture fed to milking cows, and the replacement of these lipids by an iso-dynamic amount of starch, resulted in a marked loss in milk yield. These results have been confirmed by twelve additional experiments with cows and goats, which are cited and summarized in the two most recent publications (Maynard et al., '39, '41). These publications also refer to several studies which have been made of the physiology involved. Both to ascertain whether the same results would be obtained with species other than herbivora, and also to use a species in which the purified diet technique might be employed, studies were begun with rats. Two experiments are here reported.

In both experiments, the mother rats had been fed upon our stock diet [1] from weaning, as well as through gestation. After the birth of the young, pairs of mothers were chosen which were equal in weight, and from whose litters six young for paired groups of nearly equal body weight could be selected. Whenever possible these paired groups were equalized as to sex. One mother was then fed the high-fat diet, and the other, the low-fat diet, equalizing the calorie intake in accordance with the appetite of the one consuming the

[1] The composition of this diet is given in table 1, since it was used also as the low-fat diet in the first experiment.

least amount. This proved to be the animal on the low-fat diet in practically all cases. The young were weighed daily for 17 days to provide growth records during the period when they were entirely dependent upon mothers' milk, thus furnishing a measure of lactation performance. Weight changes in the mothers were also recorded. At the end of the 17 days the young were killed and analyzed to ascertain whether any differences in growth weight were associated with differences in tissue, especially calories stored. No mothers who bore litters of less than six young were used in the experiments, and if, due to accident or other cause, the entire group of six failed to survive the 17-day period, all records for the pair of mothers concerned were discarded.

Carcass analyses on the 17-day-old young included determinations of moisture, fat and ash. Protein was calculated by difference. Immediately after killing the young by ether inhalation, the gastrointestinal tract was removed from each animal. The combined weight of each litter, after removal of the gastrointestinal tracts, was recorded as the weight of the fresh carcasses, and this value was used in the calculations of carcass composition. The carcasses were frozen and then ground preparatory to drying. The drying was carried out in vacuo at 97°C. under an atmosphere of CO_2 and was considered complete when the change in weight was not more than 0.1%.

The material here reported as fat is ether extract obtained as follows: The ground and dried carcasses were extracted with a 4 to 1 mixture of absolute ethyl alcohol and anhydrous ethyl ether. The boiling solvent was in direct contact with the dried carcass material. The total extraction time was 16 hours and 2 portions of solvent were used. The alcohol-ether extract was evaporated to near-dryness on a water bath and the final drying was accomplished in vacuo under CO_2. The resulting material was re-extracted with ether, filtered, evaporated to dryness under CO_2 and weighed.

Ash was determined on the dried defatted carcasses which had been pulverized in a power mill. Two-gram samples were ashed at 560° for 20 hours.

First experiment

In the first experiment our stock diet, containing 4.5% fat, was compared with the same diet modified to contain 9.29% of this nutrient. The composition of these diets is given in table 1. It is seen that the modification consisted of the substitution of 7% of corn oil (crude) and 1% of casein for 8% of the corn meal. The data for calorific value shown in the table were calculated by the use of the factors: 4 Cal. per

TABLE 1

Composition of diets.

NATURAL FOODS (EXPERIMENT 1)		
Constituents	Low-fat diet	High-fat diet
Linseed meal	15	15
Yellow corn meal	20.875	12.86
Ground malted barley	10	10
Wheat red dog flour	22	22
Oat flour	15	15
Dried skimmed milk	12	12
Soluble blood flour	3	3
Salt	1	1
Steamed bone meal	1	1
Cod liver oil [2]	0.125	0.14
Casein	–	1
Corn oil	–	7
	100.000	100.00
Protein (%)	23.83	22.13
Fat (%)	4.50	9.29
Cal. per gram	3.30	3.70

PURIFIED INGREDIENTS (EXPERIMENT 2)		
Constituents	Low-fat diet [1]	High-fat diet
	(*parts*)	(*parts*)
Casein	20	20.00
Yeast, ether extracted	5	5.00
Cooked starch	40	25.00
Sucrose	30	11.25
Steamed bone meal, ether extracted	2	2.00
Hawk and Oser mineral mixture	3	3.00
Oil	–	15.00
	100	81.25
Protein (%)	22.32	27.47
Fat (%)	0.03	18.46
Calcium (%)	0.95	1.16
Phosphorus (%)	0.71	0.88
Cal. per gram	3.57	4.42
Protein per 100 cal. (gm.)	6.25	6.21
Calcium per 100 cal. (gm.)	0.26	0.26
Phosphorus per 100 cal. (gm.)	0.19	0.19

[1] To each kilogram of this ration were added 14 mg. calcium pantothenate, 2 mg. pyridoxine and 1 gm. choline hydrochloride.

[2] The cod liver oil used contained 800 A.O.A.C. chick units of vitamin D and 6000 I.U. of vitamin A per gram.

gram for protein and carbohydrate and 9 Cal. per gram for fat. On the basis of the relationship thus obtained, 0.89 gm. of the high-fat diet was fed for each gram of the low-fat mixture. On this basis the intakes of protein and minerals were actually lower on the high-fat diet. Presumably they were not sufficiently lower to make any practical differences, but it was apparent that any differences in the two diets other than the fat tended to favor the low-fat mixture.

The rats with their litters were housed in cages with wire screen bottoms, equipped with feed cups to avoid feed wastage. Any wastage which did occur was accounted for, and adjustments in later intakes were made accordingly so as to keep the actual consumption equalized in terms of calories. The mothers used were approximately 12 months of age and had previously reared several litters successfully. The data for the mothers which reared their six young during the entire 17-day period with substantially equal calorie intakes are presented in table 2. Some additional pairs were removed from the experiment when the death of one of the young spoiled the comparison. A study of the incomplete records of these pairs failed to indicate that these losses of young were any larger on one diet than on the other.

The data for calorie intake in table 2 reveal that success was achieved in equalizing this intake within pairs, the largest difference for any pair being approximately 0.5%. The weights of the paired mothers show a similar degree of correspondence. It is noted that larger differences existed between the weights of the paired litters at the start, as was to be expected. The data as a whole, however, reveal that neither ration had any significant advantage in this respect. For example, the largest difference in favor of the low-fat diet is shown by pair I, and this difference is balanced by one in the other direction shown for pair K.

The data for litter gains show that in thirteen out of fifteen comparisons they were larger for the high-fat diet. They averaged 126 gm. for the latter as compared to 112 gm. for the low-fat diet. As computed by Student's ('25) method,

TABLE 2

Comparisons of low-fat and high-fat diets of natural foods.

PAIR AND LITTER	DIET	CALORIE INTAKE	WEIGHT OF MOTHER		WEIGHT OF LITTER		CARCASS ANALYSIS OF LITTERS				
			Initial	Change	Initial	Gain	Dry matter	Fat	Ash	Protein	Calories
			gm.	*gm.*	*gm.*	*gm.*	%	*gm.*	*gm.*	*gm.*	
A-66	L.F.	1137	314	+13	31.0	118.0	24.5	8.58	4.92	19.8	193
A-65	H.F.	1134	314	—23	33.5	141.5	28.7	17.01	3.80	24.8	301
B-10	L.F.	1214	279	+ 2	34.5	112.5	27.0	12.75	3.26	20.5	236
B-2	H.F.	1212	278	—16	34.5	133.5	27.7	14.84	3.44	24.4	278
C-75	L.F.	1091	245	—23	39.0	104.5	24.8	7.42	3.36	21.7	193
C-74	H.F.	1088	246	—33	35.5	121.5	28.2	13.21	3.61	24.1	261
D-0	L.F.	1203	230	+18	38.5	120.5	25.4	10.22	3.54	22.8	225
D-8	H.F.	1200	227	—13	40.5	140.5	28.5	15.56	4.10	27.4	302
E-2	L.F.	863	264	—15	33.5	87.0	24.0	6.70	2.69	17.2	161
E-40	H.F.	861	261	—48	33.5	93.5	25.4	7.78	2.98	18.6	179
F-69	L.F.	1289	295	+ 6	33.0	136.5	26.6	13.60	3.89	23.3	260
F-31	H.F.	1286	300	—28	38.5	152.5	28.0	17.64	3.92	27.0	319
G-84	L.F.	1175	276	+14	35.0	121.0	25.6	9.99	3.81	22.8	223
G-87	H.F.	1172	271	—11	40.5	132.5	26.5	13.29	3.86	26.0	273
H-62	L.F.	978	276	—41	37.0	102.0	24.4	7.02	3.44	20.8	184
H-9	H.F.	976	292	—43	37.5	126.5	28.7	15.34	3.43	24.6	284
I-74	L.F.	1026	247	—17	47.0	106.0	25.0	8.04	3.75	23.0	206
I-77	H.F.	1031	222	—36	37.0	125.0	28.6	24.44	2.39	16.1	322
J-1	L.F.	873	282	—19	35.5	97.5	24.2	7.39	2.96	18.5	174
J-33	H.F.	871	282	—44	33.0	90.5	23.9	5.97	2.66	18.0	158
K-87	L.F.	838	254	—38	32.0	77.0	23.3	5.37	2.47	15.3	137
K-7	H.F.	836	256	—59	44.0	78.0	25.1	5.80	2.93	19.4	164
L-68	L.F.	1279	315	0	37.5	129.0	27.2	14.18	3.62	23.2	265
L-66	H.F.	1248	316	+ 1	35.0	144.0	26.9	13.22	3.85	25.8	271
M-68	L.F.	1544	311	+10	36.5	163.5	28.6	15.94	4.27	31.8	330
M-69	H.F.	1469	305	+ 7	35.5	166.5	29.1	20.81	*	*	*
N-9	L.F.	1173	280	+ 8	36.0	134.0	26.6	13.80	3.64	23.7	264
N-1	H.F.	1144	291	— 9	33.0	132.0	28.3	15.08	*	*	*
O-70	L.F.	870	289	—45	41.5	68.5	23.2	4.75	2.68	16.0	135
O-32	H.F.	868	299	—67	38.0	112.0	25.7	8.99	*	*	*

* Carcass samples lost before data obtained, due to an explosion.

the odds in favor of the results being significant are 1249 : 1. The daily weight records (not shown) indicated that the more rapid growth of the litters from the mothers receiving the high-fat mixture became evident beginning at about the eighth day. That the gains in body weight reflected actual differences in tissue substance is indicated by the data for the percentage of dry matter in the carcass. These data show that in thirteen out of fifteen cases the young reared on the high-fat diet contained a larger percentage of dry matter, with calculated odds greater than 4999 : 1 in favor of this result. The evidence here given is further borne out by the other data for carcass composition. In thirteen out of fifteen comparisons, the young from the mothers receiving the high-fat diet contained more fat, the litters on this diet averaging 13.9 gm. compared to 9.7 gm. for the low-fat diet. The odds in favor of the significance of the difference are 356 : 1. The figures for protein favor the high-fat diet in ten out of twelve cases for which the data were obtained. Here the odds were 27 : 1. The figures for ash are variable and show no significant trend, although they favor the high-fat mixture in the majority of the cases.

The calorific values of the carcasses were calculated to give a single measure of the differences in energy stored on the equi-caloric intakes. These values were obtained by multiplying grams of carcass fat by 9.45 and grams of protein by 5.65, representing the average heats of combustion of these nutrients. The data reveal an advantage for the high-fat diet in eleven out of the twelve comparisons for which data were available for the calculation. The odds in favor of the significance of this difference are 999 : 1. On the average the litters from the mothers receiving the high-fat diet contained approximately 25% more calories than their pair mates.

Certain animals were used twice in experiment 1, being placed first on one diet and then on the other. A-66, which received the low-fat diet and performed less satisfactorily in all respects than her pair mate, later received the high-fat diet as L-66 and excelled her pair mate. Similarly B-2, which excelled on the high-fat diet, proved inferior to her pair mate

when later, as E-2, she received the low-fat mixture. Insofar as the data were obtained the same was found true for the animal which appears once as G-87 (high-fat) and again as K-87 (low-fat). These comparisons suggest rather clearly that the uniformly better performance on the high-fat diet could not have been due to the chance selection of animals of higher productive capacity for that diet. The significance of the results is strengthened thereby.

In connection with differences in calories produced, attention should be given to the column of figures in table 2, showing the changes in the weights of the mothers. It is noted that losses in weight occurred in the majority of cases and that some very large losses are recorded. One would expect such losses to be more likely to occur under the limited feeding system imposed by the paired feeding technique. Of more significance, however, the decreases in weight are greater (or the gains are less) for the mothers on the high-fat diet in all cases except one. On the average the mothers receiving this diet revealed an average net loss of 18.6 gm. greater than the mothers on the other diet. This means that whereas the high-fat diet clearly resulted in a larger lactation performance, this performance occurred in part at least at the expense of body reserves. Without knowing the composition of the tissues lost by the mothers, it is impossible to make any estimate of the relationships involved. Citing an extreme case, animal O-32 lost 67 gm. while nursing a litter which gained 112 gm., whereas her pair mate lost 45 gm. nursing a litter which gained 68 gm. Though it lost more body tissue, the much larger production performance of the high-fat mother is evident. The equalized feeding technique does not disclose whether, if the high-fat diet had been fed ad libitum, smaller losses would have occurred accordingly. This question was studied later as is reported under experiment 2. In looking over the data as a whole one notes that for certain pairs the gains of the litters markedly exceeded those of other pairs. These differences are roughly correlated with differences in

calorie intake, and presumably individual appetites are here concerned.

The average weights at 17 days of all the young reared by the mothers on the low-fat and high-fat diets were 24.7 gm. and 27.1 gm., respectively. These figures, particularly the latter, represent excellent weights for the average of a litter of six at 17 days in our colony. They indicate that the comparison was made at a high level of performance.

Second experiment

In this experiment a purified diet designed to be very low in fat (actually 0.03% of ether extract) was selected and modified by the substitution of 15 parts of either crude coconut oil or crude corn oil for an equal amount of starch to provide the high-fat ration. These diets are shown in table 1. The calorific values per gram are calculated as described in experiment 1. On the basis of these calculations the calorific intakes per pair were equalized by feeding approximately 0.8 gm. of the high-fat diet for each gram of the low-fat mixture. It is noted that whereas the high-fat diet had more protein, calcium and phosphorus on a percentage basis, both diets supplied the same amounts per 100 calories. Thus the mothers' intakes were equalized for these nutrients also, a provision which could not be met in experiment 1 without markedly altering the ingredient relations.

Each animal on the purified rations received a daily supplement of 1 drop of A-D vitamin concentrate, so diluted with cottonseed oil that each drop contained 13.8 U.S.P. units of vitamin A and 1.95 units of vitamin D. To insure an adequate supply of vitamin D, each animal received direct irradiation at a distance of 3 feet from a quartz mercury vapor lamp for a period of 5 minutes weekly. It is noted in the table that the low-fat diet was supplemented with choline, pantothenic acid and pyridoxine. The choline was added to make sure that any value obtained from the added fat was not due to this constituent. It was planned, considering the possibility of additional needs, to add the other vitamins to both diets, but due

to an error they were omitted from the high-fat diet. Thus if these added vitamins were of benefit, the low-fat mixture was favored thereby. Despite these additions it cannot be said, particularly in view of the recent work of Sure ('41 a, '41 b), that either diet was optimum in all vitamins required for lactation. It is considered, however, that the content of the known vitamins of the low-fat diet was equal to that of the high-fat diet. Further, it should be borne in mind that the rats received a natural diet up to the time of parturition and that the purified diets were fed for only 17 days. The questions here involved are now under study, as is referred to later.

The general plan of the experiment was the same as for the one previously described. At the outset, females approximately 1 year of age which had previously produced several litters were used, but it was found impossible to obtain satisfactory food consumption, or even litter survival by shifting them suddenly to the purified rations following parturition. After failures with approximately fifteen of these females, young females about 90 days of age were used and successful comparisons were obtained. The data are presented in table 3 for eleven pairs of females. For five of these pairs the high-fat ration contained coconut oil and for the others it contained corn oil. Both oils were used in order to ascertain whether the wide differences in fatty acid distribution here represented might be reflected in differences in lactation performance.

It is noted in table 3 that for each pair, success was achieved in selecting mothers of comparable weight and in equalizing their calorie intakes. It is also noted that any variations in the initial weights of the litters were less than in experiment 1. The gains of the litters are in favor of the high-fat diet in ten out of eleven cases, by odds of 73:1, averaging 122 gm. for the low-fat diet and 138 gm. for the other. The differences here involved represent an actual increase in dry matter as is indicated by the percentage figures for the latter. The odds here are 1249:1. In all comparisons the high-fat diet resulted in more fat in the litters, the litters on the high-fat diet

TABLE 3

Comparisons of low-fat and high-fat purified diets.

PAIR AND LITTER	DIET	CALORIE INTAKE	WEIGHT OF MOTHER		WEIGHT OF LITTER		CARCASS ANALYSIS OF LITTERS				
			Initial	Change	Initial	Gain	Dry matter	Fat	Ash	Protein	Calories
			gm.	*gm.*	*gm.*	*gm.*	%	*gm.*	*gm.*	*gm.*	
A-59	L.F.	1196	187	+12	31.5	91.0	28.3	11.91	2.76	16.7	207
A-8	H.F.[1]	1196	190	— 5	31.0	119.5	28.0	13.80	4.46	20.0	243
B-13	L.F.	1257	231	+ 2	37.5	134.5	26.1	12.03	4.30	25.5	258
B-12	H.F.[1]	1260	237	—14	35.5	146.5	33.2	25.69	3.81	25.5	387
C-17	L.F.	1190	201	+ 9	34.5	109.5	25.2	9.76	3.18	20.2	206
C-4	H.F.[1]	1183	194	— 1	35.0	121.0	28.7	14.88	3.42	22.2	266
D-14	L.F.	1385	240	0	33.5	149.5	29.8	19.14	4.17	26.2	329
D-17	H.F.[1]	1389	236	—10	33.5	174.0	32.5	26.20	4.32	32.0	428
E-3	L.F.	816	220	—30	37.5	94.0	23.8	7.33	3.03	18.2	172
E-1	H.F.[1]	818	228	—28	39.5	107.5	27.3	11.85	3.21	21.2	232
F-29	L.F.	1498	242	+44	36.5	132.5	28.3	16.90	3.51	22.3	286
F-3	H.F.[1]	1501	250	0	37.0	173.0	30.8	23.12	4.31	32.5	402
G-16	L.F.	1519	216	+21	32.0	158.0	28.7	17.27	4.19	28.1	322
G-12	H.F.[2]	1523	216	— 8	34.5	141.5	30.3	19.48	4.07	24.6	323
H-61	L.F.	1128	206	— 2	30.5	110.5	25.7	9.76	3.37	20.0	205
H-19	H.F.[2]	1131	261	—32	35.0	116.5	29.3	16.09	3.38	21.7	275
I-19	L.F.	1014	264	—34	32.5	80.5	26.2	8.08	2.61	16.0	167
I-13	H.F.[2]	1017	261	—42	35.5	125.5	27.6	13.07	*	*	*
J-59	L.F.	1349	236	— 6	37.0	152.0	28.9	16.95	*	*	*
J-16	H.F.[2]	1353	250	—15	35.0	155.0	30.2	21.52	3.92	26.7	354
K-17	L.F.	1291	230	0	38.5	135.0	27.3	13.63	3.73	25.6	273
K-18	H.F.[2]	1294	233	—17	34.0	141.0	30.8	20.67	3.55	25.1	337

[1] Fat supplied by corn oil.
[2] Fat supplied by coconut oil.
* Carcass material lost before data obtained.

averaging 18.8 gm. and those on the other, 13 gm., the odds being over 4999:1. This is true for protein in six out of the nine cases for which data are available, one pair showing no difference in either direction. But here the odds are only 9.5:1. The ash data favor this same diet in six out of nine cases. The figures for calories favor the high-fat ration in all comparisons, with odds of 1249:1. On the average, the litters from the mothers receiving the high-fat diet contained approximately 28% more calories than those from the other mothers. The figures do not reveal any significant differences between the performances of the mothers on the high-fat diet where the oils were different. A more critical experiment would be needed to demonstrate any differences which may exist.

The young from all the mothers receiving the low-fat diet averaged 26.1 gm. body weight at 17 days, and those produced on the high-fat mixture averaged 27.4 gm. Like the first experiment, these data show that the comparison was made at a high level of performance.

Also like the first experiment, the increased lactation performance on the high-fat diet was accompanied by a larger loss (or a lesser gain) in the mothers' reserves, as is shown by the data for change in their weight. On the average the mothers receiving the low-fat diet gained approximately 1 gm. while those on the high-fat mixture lost 16 gm. In order to ascertain whether this loss was due to the restricted feeding, five mothers with new born litters of six were placed on the high-fat (corn oil) diet and fed ad libitum for 17 days. The results were as follows:

MOTHER NO.	WEIGHT CHANGE OF MOTHER	AV. WEIGHT OF YOUNG AT 17 DAYS
	gm.	*gm.*
49	+ 7	33
181	+14	34
131	+12	33
141	+20	35
97	+ 4	39

These data show that with ad libitum feeding the high-fat diet both caused the mothers to gain in weight instead of lose and to surpass the previous performance for milk production. The latter is indicated by a comparison of the data for average weight of young at 17 days with the average figure of 27.4 gm. obtained with the restricted feeding. Clearly the results produced with the high-fat diet fed ad libitum represent a remarkable lactation performance.

The similar results of two experiments with different diets furnish very strong evidence that the substitution, on an isodynamic basis, of fat for a part of the carbohydrate in a low-fat diet results in a higher lactation performance. This is in accord with the previous findings of this laboratory with cows and goats. Various possible explanations, such as more efficient metabolism of fat calories for fat secretion, need for specific fatty acids, vitamin relationships, and others, have been discussed in previous reports. The present experiment provides no answer here other than revealing no significant difference between two fats of widely different fatty acid distribution. The fact that the larger lactation performance in the rats fed the diet of higher fat content was accomplished in part at least at the expense of the mothers' reserves might suggest some specific stimulating action. The need is recognized for a repetition of the work with more attention to all of the vitamins required for lactation in the rat. Specifically it is possible that on the low-fat diet there was a relative shortage of thiamine for the metabolism of its higher carbohydrate content. Calculations suggest that this possibility is an unlikely explanation of the results obtained but an experiment to test this question is now in progress.

While the differences in favor of the high-fat diet appear convincing and while no evidence of loss of the mothers' reserves was noted with ad libitum feeding despite a further increase in lactation performance, these results by themselves should not be interpreted to apply to other species. The very

different composition of rat milk, particularly its fat content, must be kept in mind. The principal contribution of the present experiment is to provide a technique by which the various physiological questions can be studied to better advantage than in the cow or goat. Such studies are being planned.

SUMMARY

Paired-feeding studies, involving equalized calorie intakes, are reported in which lactation performance was measured by the growth of standardized litters and by their composition. In one experiment a diet of natural foods containing approximately 4.5% fat was compared with a similar diet containing approximately 9% fat. The young from the mothers on the high-fat diet made better growth and contained more dry matter and fat in thirteen out of fifteen paired comparisons. Similar results were obtained for protein and calorie content. In a second experiment in which purified diets containing 0.3% and 18% fat were compared the data for the high-fat diet indicated a superiority in growth and in dry matter content in ten out of twelve comparisons, in fat and calorie content in all cases, and in protein in six out of nine cases. A statistical analysis indicated a high degree of significance for the results of both experiments. With the limited feeding practiced, the better lactation performance on the high-fat diet occurred in part, at least, at the expense of the mothers' reserves.

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ASCORBIC ACID EXCRETION AT KNOWN LEVELS OF INTAKE AS RELATED TO CAPILLARY RESISTANCE, DIETARY ESTIMATES, AND HUMAN REQUIREMENTS [1]

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INTRODUCTION

The literature concerning the significance of vitamin C in human nutrition has been reviewed by Bessey ('38), King ('38), Smith ('38) and Todhunter ('40). Several types of data have been used as a basis for estimating requirement including (1) urinary ascorbic acid, (2) capillary fragility, and (3) ascorbic acid in blood, all at different levels of intake and under a variety of conditions. Kellie and Zilva ('39) reported that a minimum intake of 30 to 40 mg. of ascorbic acid is necessary to produce a standard response to a "saturation" dose of 600 mg. ascorbic acid. Belser, Hauck and Storvick ('39), using a 400 mg. saturation dose, found that five women subjects required 70–100 mg. and two men needed 110–135 mg. of ascorbic acid intake daily to maintain saturation. Seventy to 90 mg. of ascorbic acid was necessary to keep three women saturated according to Todhunter ('40). Ralli, Friedman and Sherry ('39) observed no clinical symptoms of vitamin C deficiency in three men on a 50 mg. intake but observed no increase in urinary excretion until the intake reached 100 mg. and the blood plasma level reached 0.1 mg.

[1] Contribution no. 607 of the Rhode Island Agricultural Experiment Station.

[2] A portion of the data was presented by Miss Tatiana Levcowich in partial fulfillment of the requirements for the degree of Master of Science at Rhode Island State College, June, 1939.

A study of the urinary ascorbic acid excretion and capillary resistance of college women (Mitchell, Merriam and Batchelder, '38) has led to further investigation at Rhode Island State College of the vitamin C status and ascorbic acid requirement of such women.

EXPERIMENTAL

Student diets and excretions-survey study. A 1938 study of Rhode Island State College women, for whom results for 1937 have already been reported (Mitchell, Merriam and Batchelder, '38), showed an average urinary ascorbic acid excretion from thirty-two women of 24 mg. (range 5–122 mg.) as compared with 17 mg. (range 5–56 mg.) for the same subjects the previous year. The 1938 average ascorbic acid intake was 122 mg. (range 56–253 mg.) as compared to 81 mg. (range 32–126 mg.) in 1937, when calculated from a dietary record for the 7 consecutive days preceding and including the 24-hour urine collection. The coefficient of correlation of the intake and output of ascorbic acid for all 1938 studies was +0.48. This was slightly higher than that for the previous year. The coefficient of correlation of the urinary ascorbic acid excretions with that of the same subjects tested the year before and already reported was +0.45. Although the absolute values of the calculations of intake are open to question as discussed later, increased urinary output shows a consistent relation to increased intake.

Studies were made on the urinary ascorbic acid excreted by eight women under various conditions: (1) when diets were freely chosen; (2) when ascorbic acid intake was increased through ingestion of large quantities of citrus fruits and vegetables; (3) when ascorbic acid in the diet was reduced to a minimum; (4) when massive doses of crystalline ascorbic acid were given; and (5) when ascorbic acid was given at various test levels for determination of dietary needs. Capillary resistance tests were made while the subjects were on different levels of known ascorbic acid intake.

The college medical office classified these women, through medical examination, x-ray, and urine analysis, as healthy and capable of carrying on the usual college activities.

The methods for determining ascorbic acid and capillary resistance were the same as those previously described for the Rhode Island studies by Mitchell, Merriam and Batchelder ('38). For the last two subjects on whom most of the studies have been made, diets averaging not more than 5 mg. ascorbic acid were prepared in a special kitchen and were planned to meet present dietary standards for proteins, minerals, and vitamins. In order to assure optimal intakes of the vitamins

TABLE 1

Urinary excretion of ascorbic acid as related to estimated intake from freely chosen diet and to intake of crystalline ascorbic acid.

NO. OF DETERMINATIONS	INTAKE ESTIMATED		EXCRETION	
	Average	Range	Average	Range
	mg.	*mg.*	*mg.*	*mg.*
29	87	27–193	13	6–16
27	121	44–253	22	18–29
	Intake crystalline ascorbic acid			
	50 [1]		22	17–29

[1] Plus 5 mg. in foods included in the diet.

A, D, and the B complex, dry yeast and halibut liver oil were given daily. For the earlier subjects, the diets were planned on the basis of foods available at the college cafeteria and supplemented with yeast and haliver oil as mentioned above. The results indicate that this arrangement provided a satisfactory vitamin C free diet.

Excretion at different intake levels. Excretions of subjects at a 50 mg. intake level are used to interpret the results of the student diet studies as shown in table 1. The excretion of the crystalline ascorbic acid group was measured during a post-saturation period. The excretion of the survey group was measured at the customary level of intake and is pre-

sumably characteristic of that intake. Even though daily fluctuation may be a source of error in individual cases, the results for the whole group are probably representative.

The members of the survey group never excreted as much ascorbic acid as the group receiving 50 mg. daily in a post-saturation period. This indicates that their habitual daily intake (although high according to calculation) was actually quite low.

The evidence leads us to conclude that excretions on known intakes of 50 mg. crystalline ascorbic acid plus an average of 5 mg. taken in the basal diet are comparable to excretions on freely chosen diets which, by calculation, would appear to contain at least twice as much as the amount excreted. Most of the subjects on the freely chosen diets were eating in a college cafeteria. Comparison of excretions on a known intake and on a calculated intake indicates that there is danger of overestimating the actual vitamin C content of diets, particularly where food is prepared and served under conditions which may contribute to the destruction of vitamin C. From a practical standpoint, it may be desirable, therefore, to publish popular food guides which are specific as to the kind and amount of vitamin C food required. Important factors such as the length of time between harvesting and eating, storage conditions, and methods of cooking may reduce the vitamin C in a food to a point far below that shown in the most extensive of tables.

Capillary fragility as related to character of diet. In the light of new reports concerning capillary fragility as affected by vitamin P as well as vitamin C, capillary resistance tests (Dalldorf, '33), were resumed when subjects M. A. and R. B. were observed at various known levels of crystalline ascorbic acid intake (table 2).

An average of at least 15–20 cm. negative pressure was necessary to produce petechiae on the front of the arm for both subjects. A similar average was obtained in the group studied in 1937. Capillary resistance was not significantly

affected by varying the amount of crystalline ascorbic acid added to the vitamin C-free diet. The capillary resistance values on the back of the arm were, however, consistently higher in both subjects. The average was 30–35 cm. negative pressure. This differs from the results reported by Anderson,

TABLE 2

Average daily ascorbic acid excretion at different levels of intake as related both to excretion following a 400 mg. dose and to capillary resistance.

SUBJECT	ASCORBIC ACID INTAKE	EXCRETION		CAPILLARY RESISTANCE [2]			
				Front of arm		Back of arm	
		During test period [1]	Following 400 mg. dose	Test period	After 400 mg.	Test period	After 400 mg.
	mg.	mg.	%	cm.	cm.	cm.	cm.
M. A.	40	18	16	10	15	15	20
	50	24	28	15	15	40	40
	50	20	33	20	20	35	35
	110	62	42	20	15	40	40
	120	33	30	15		40	
R. B.	40	19	26	10	15	20	25
	50	22	28	15		25	
	50	23	18	15	15	30	30
	60	34	27	15	15	30	30
	70	32	24	15	20	30	30
	90	55	32	15	15	35	30
	110	41	43	20	15	30	35
	120	50	32	20	20	40	30
	130	73	47	25	25	40	40
	140	71	21	25		40	
	140	69	57	20		30	

[1] Average of last 3 days of each 6-day test period.

[2] Negative pressure necessary to produce petechiae.

Hawley and Stephens ('36) who found only 1–2 cm. greater negative pressure necessary for the outer arm as compared to the inner arm.

In the nineteenth week of experiment the petechiae resulting from the test, although appearing at the same pressure and in the same numbers, began to increase in size and were so much

larger than those previously observed that the subjects made comments regarding the change. The petechiae of subsequent tests continued to remain large until the Easter recess, during which the subjects returned to freely chosen diets high in vitamin C rich foods. Upon the return to Campus when M. A. was tested the petechiae had returned to smaller size. Although no test could be made on R. B. until 4 days after she had returned to the basal diet plus crystalline ascorbic acid, similar, but not as striking, results were observed. About 2 weeks later the petechiae began increasing in size again.

These observations are similar to those of Scarborough ('39) who reported that conflicting evidence by previous observers might be accounted for by the fact that vitamin P, as well as vitamin C, affects capillary fragility. Although our evidence is limited, it seems possible that the large petechiae of subjects on our vitamin C free diet plus crystalline ascorbic acid might be evidence of lack of another factor ordinarily ingested when the vitamin C of the diet is taken in the form of natural foods. In our two subjects a few days on natural foods appeared to result in smaller petechiae. This may be evidence of a lack of vitamin P in the foods included in the vitamin C free diet. Further study is needed in this connection.

Excretion on vitamin C-free diet. Table 3 summarizes the urinary ascorbic acid data of three subjects on the vitamin C deficient diet (average ascorbic acid content, 5 mg. daily).

TABLE 3

Ascorbic acid excretion on a vitamin C-free diet.

SUBJECT	DAYS ON DIET	DAILY EXCRETION	
		Average	Range
		mg.	*mg.*
T. L.	1	36	
	2– 8	13	11–16
	9–13	10	9–11
C. B.	1	16	
	2– 9	11	7–16
M. A.	1	26	
	2– 7	16	12–19

after long continued high intake. Excretion for the first 24 hours on the deficient diet fell sharply as compared to that for the previous day. By the second day, the excretion had fallen to a characteristic resting level similar to that reported by O'Hara and Hauck ('36).

During the first day subjects T. L., C. B., and M. A. excreted 36, 16, and 26 mg. respectively; while during the following periods of 7, 8, and 6 days respectively the excretions averaged 13, 11, and 16 mg. During an additional 5 days on the diet, T. L. excreted an average of 10 mg. Apparently the exact level of previous vitamin C intake has little effect on the excretion after 1 day on a diet deficient in this factor. The apparent vitamin C content of the urine, however, is maintained at a somewhat higher level than the intake, perhaps because appreciable amounts of. other reducing substances are included in this figure. This was suggested by Ralli, Friedman and Sherry ('39), whose results by titration were slightly higher, and by photoelectric colorimeter somewhat lower than ours. It may, however, be due in part to a continuous loss of vitamin C from the tissues.

Excretion as indicative of requirement. The records for subjects M. A. and R. B. at different levels of intake following "saturation" as interpreted by O'Hara and Hauck ('36) are summarized in table 2. After the end of each test level the subjects were fed high vitamin C, usually 200 mg., until a 50% excretion of a 400 mg. dose was observed. M. A. tended to take longer to reach this "saturation" level after each test period than did R. B. For that reason it was not possible to test her at all levels observed for R. B. during the same period of time. The excretion at increasing levels of intake showed a tendency to rise, but neither these figures nor the percentage excretion of a 400 mg. dose following each period on a test level showed sufficiently consistent and regular increments to justify a conclusion that the higher levels were of specific advantage. Ralli, Friedman and Sherry ('39) raised the question, in connection with blood plasma levels, whether intakes necessary to give maximum value for ascorbic

acid content of blood and tissues are essential even though they may be desirable. We have no subjective nor objective evidence of ill-effects either at the lowest or at the highest levels of ascorbic acid fed. There is no evidence that the body, after a period on low as compared with high ascorbic acid intake, needed to accumulate more total ascorbic acid before saturation was reached.

Subjects M. A. and R. B. had a basal energy metabolism, 10 and 17% respectively, below the Aub and Dubois standards as modified by Boothby and Sandford. In the light of the close relation of ascorbic acid to oxidation-reduction processes in the body, it seems reasonable to assume that an individual's requirement may vary with the total energy metabolism. It is possible also that a slight additional loss may be due to excessive perspiration resulting from heat or unusual activity as reported by Bernstein ('37). On the basis of these observations and in the light of other less complete studies, it seems safe to conclude that the minimal ascorbic acid requirement for fairly active college women may lie at about 50 mg. daily. A dietary standard or allowance for ascorbic acid estimated with the safety factor of 50%, commonly used for many nutrients, would then be 75 mg.

As previously discussed, this figure is based on studies with crystalline ascorbic acid. If it is to be applied to estimates of intake on freely chosen diets it should probably include only foods such as freshly expressed citrus fruit juices, freshly boiled or baked potatoes, and other products not likely to have suffered serious losses of vitamin C due to local differences in variety or method of preparation. An example of such differences is reported by Murphy ('41).

SUMMARY AND CONCLUSIONS

Certain aspects of vitamin C metabolism have been studied in forty-five young women during a survey study and during nine intensive control periods. Capillary fragility and urinary ascorbic acid, as observed when the ascorbic acid intake was estimated from freely chosen foods and when crystalline

ascorbic acid was added to a low vitamin C diet at various known levels, have been reported and discussed. Conclusions based upon observations made during the investigation are:

1. The calculated ascorbic acid content of the foods in a freely chosen diet tends to be overestimated, and may lead to a false sense of security as to vitamin C intake, unless very specific food guides can be set up.

2. Capillary resistance is not significantly affected by variation in known crystalline ascorbic acid intake. More negative pressure, however, is necessary to produce petechiae on the back of the arm than on the front. Over an extended period on a crystalline ascorbic acid intake the petechiae become larger in size, but do not change in number. This suggests that the vitamin C-free diet used here may lack vitamin P or some other factor affecting capillary fragility.

3. Urinary ascorbic acid excretion during a vitamin C deficient diet drops sharply on the first day, and on the second day reaches a fairly constant resting level for all subjects.

4. For moderately active young women a minimal requirement of 50 mg. and a dietary standard (or allowance) of 75 mg. daily appears satisfactory.

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STUDIES OF LIBERAL CITRUS INTAKES. I.[1]

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The literature affords several instances of attempts to study the tolerance of the human body for fruit juices rich in citric acid. In many of these (for example, Blatherwick and Long, '22; Saywell and Lane, '33) the amount of citric acid which escaped oxidation was computed indirectly from the "total organic acids" of the urine, on the presumption that the rise in this value when citrus juices are taken is wholly due to, and fully reflects, any "extra" excretion of "unburned" citric acid. That both aspects of this assumption are unjustified was demonstrated in an earlier study (Sherman, Mendel and Smith, '36 b), by direct determination of citric acid side-by-side with titration of "total organic acids" under various conditions.

However, in the carefully controlled studies of Schuck ('34) on six young women given 1000 cc. daily of orange juice, the urinary excretion of citrate was determined directly during both control and experimental periods, each of several days' duration. In each case as reported by Schuck, the excretion of citric acid was greater on the days when orange juice was taken, the increase of daily elimination for her different subjects ranging from 0.234 gm. to 1.294 gm. of citric acid, and corresponding to 2.6 to 16.6% of the citric acid contained in the fruit juice (7.95 to 8.51 gm.). With the diet maintained constant otherwise, orange juice ingestion invariably de-

[1] The aid of a grant from the Florida Citrus Commission is gratefully acknowledged.

creased the acidity of the 24-hour urine sample of Schuck's subjects, the change averaging 0.9 pH unit.

With the greatly increased supply of citrus fruits during the past few years, and the growing tendency to commend them to an ever more prominent place in the dietary, it seemed of importance to determine: (1) whether in a larger representation of the population a considerable proportion would be found to show such a relatively poor utilization of dietary citrate as the one of Schuck's subjects who eliminated one-sixth of the ingested citric acid unchanged; and (2) whether at still higher levels of consumption of citrus juices any indication of intolerance would become evident, either in subjective sensations or in the chemistry of the urine, by a greatly increased output of citric acid or a lowered pH value.

EXPERIMENTAL

The subjects of these experiments on grapefruit juice were nine young women and sixteen young men, all between the ages of 22 and 37 years. On the so-called control day, each of these subjects collected, under toluene and with the customary precautions, the 24-hour urine sample. On the following ("experimental") day, during the first 10 hours of which the subject drank 1800 cc. of fresh grapefruit juice, a second 24-hour sample was collected. A record was kept of the amounts and kinds of food consumed on both days, and from this the citric acid intake (other than from the grapefruit juice) was estimated from the figures of Hartmann and Hillig ('34). The subjects were provided with a list of foods of high citric acid content, which they were requested to avoid; but in some instances milk was taken, despite its considerable citrate content. Each of the ten subjects indicated by footnote 1 in table 1 had an identical basal diet on the control and experimental days. The basal diets of the various individuals were, however, different.

The grapefruit juice was freshly extracted for each experiment and strained through a coarse sieve. Each batch was analyzed for total citric acid by the method of Pucher, Sherman and Vickery ('36). The thirteen different samples

of grapefruit juice used in this series showed an average content of total citric acid of 1.20%, with a range from 0.82 to 1.70%. The five samples which were of the "seedless" variety had a lower citric acid content (1.01%) than did the eight samples with seeds (1.32%), obtained at the same seasons of the year.

On each 24-hour urine sample, the pH was measured, using the glass electrode[2], and the total citric acid (i.e., sum of free acid and salt forms) was determined by the method of Pucher, Sherman and Vickery ('36).

In addition to serving as subjects in this type of experiment, four individuals on another day consumed their same basal diet but, instead of drinking the grapefruit juice over a 10-hour period, took a large volume all within 15 minutes. Urine was collected during a control period of 2 hours immediately before the juice was drunk, and thereafter in four successive 1½-hour intervals, followed by a 16-hour period, each of these six samples being analyzed separately for both pH and citric acid content. The findings of these short-time experiments are to be described more fully later, and are mentioned only briefly in the present discussion.

RESULTS AND DISCUSSION

Effect of grapefruit juice on citrate excretion. In table 1 is shown for each subject the total citric acid intake on the control and on the experimental day, the 24-hour urinary excretion of citrate on the 2 days, and the difference between the latter two values expressed as a percentage of the difference between the former two values. This last-mentioned value may be regarded as representing the percentage of the extra citric acid ingested in the grapefruit juice which escaped oxidation in the body[3], if it may be assumed that the basal or

[2] All measurements of pH were made by Dr. Oscar E. Lanford.

[3] The four studies in which urinary citrate was determined in samples collected over successive short intervals of time indicate that, even when a large volume of grapefruit juice is ingested at one time, any resulting extra excretion of citrate ceases within about 6 hours. Since in the experiments summarized in table 1, the 24-hour collection terminated at least 14 hours after the last portion of juice was taken, it seems entirely probable that such samples included all of the unburned citric acid which escaped through the kidneys.

endogenous excretion of citrate was the same on the 2 days. This assumption, is, however, not altogether valid, for Sherman, Mendel and Smith ('36 a) showed that, on a diet

TABLE 1

Data for individual subjects on citric acid intake, urinary excretion of citric acid, and pH of the 24-hour urine sample.

SUBJECT	CITRIC ACID INTAKE		CITRIC ACID EXCRETION		INCREASED EXCRETION OF CITRIC ACID AS % OF INCREASED INTAKE	URINARY pH	
	Control	Exptl.	Control	Exptl.		Control	Exptl.
Males	*gm.*	*gm.*	*gm.*	*gm.*			
C. S. R.[1]	0.48	15.33	0.515	0.792	1.87	6.62	6.73
R. W. L.	1.73	21.84	0.546	1.094	2.72	6.18	7.06
R. G. L.	1.87	22.00	0.299	0.664	1.81	6.46	7.02
W. D.	0.72	16.93	0.420	0.891	2.91	6.46	7.08
H. C. K.	0.26	17.08	0.279	0.391	0.67	5.86	6.06
Z. H.[1]	0.08	19.88	0.237	0.705	2.36	5.62	6.61
R. C. B.[1]	0.29	20.09	0.354	0.676	1.63	5.40	6.12
F. E. H.	1.01	27.91	0.468	0.742	1.02	5.85	6.45
G. R. L.	1.01	27.91	0.610	1.168	2.07	5.91	6.75
E. L.[1]	0.49	17.01	0.653	0.848	1.18	6.00	6.05
F. A. F.	0.47	24.25	0.426	1.040	2.58	5.78	6.70
H. McC.	0.29	23.98	0.293	0.666	1.57	6.29	6.82
D. T. V.	0.58	25.95	0.418	0.741	1.27	5.59	6.52
H. D.	0.48	26.10	0.354	0.805	1.69	5.73	6.52
H. B.	0.50	31.11	0.296	0.818	1.38	5.99	6.74
G. H.	0.12	30.80	0.275	0.738	1.51	5.48	6.70
Mean ±	0.65	23.14	0.403±	0.802±	1.76±	5.95±	6.62±
its P. E.			0.021	0.031	0.010	0.06	0.05
Females							
M. C. U.[1]	0.36	21.96	0.731	1.469	3.42	6.22	6.75
A. B. R.[1]	0.42	15.27	0.583	1.008	2.86	6.47	6.81
M. M. W.	1.05	17.35	0.641	1.040	2.45	5.77	6.37
F. V. D.[1]	0.68	29.03	0.528	1.080	1.95	5.71	6.55
A. Z.	0.47	29.21	0.970	1.476	1.76	5.62	6.63
A. U.[2]	0.48	16.86	0.671	1.074	2.91[2]	6.05[2]	6.70[2]
A. U.[2]	0.50	17.59	0.536	1.112	2.97[2]	5.58[2]	6.28[2]
M. L.[1]	0.49	17.01	0.659	0.827	1.20	6.17	6.57
I. M. S.[1]	0.25	24.10	0.638	1.263	2.62	5.55	6.80
C. S. L.[1]	0.04	23.89	0.435	0.771	1.41	6.29	6.38
Mean ±	0.47	21.76	0.643±	1.111±	2.27±	5.94±	6.58±
its P. E.			0.033	0.055	0.175	0.03	0.04

[1] Subject had same basal diet on control and experimental days.

[2] Experiment with A. U. was repeated. The *average* value for the two experiments was used in group calculations.

essentially devoid of citric acid, the citrate excreted in the urine, which must have been of "endogenous" or "metabolic" origin, increased in quantity with a shift of the body's acid-base equilibrium in the direction of alkalosis. Since consumption of grapefruit juice resulted in all cases in raising the urinary pH to a greater or less degree a correspondingly larger or smaller fraction of the increased citrate elimination is to be attributed to this circumstance. Therefore, although it is convenient to speak of the percentages in table 1 as that part of the citric acid of the grapefruit juice which the body "could not" ("failed to") oxidize, it should be remembered that as such they are somewhat too high.

With this reservation it may then be pointed out in summary that, of the citric acid contained in 1800 cc. of grapefruit juice, the sixteen men of the present study failed on the average to metabolize $1.76 \pm 0.010\%$, while in the case of the nine women the fraction escaping oxidation averaged $2.27 \pm 0.175\%$. Conversely, considering both sexes together, it thus appears that on the average at least 98% of the citric acid supplied in the grapefruit juice was utilized under the conditions of this experiment. In its availability to the body as a source of energy, citrate thus compares favorably with the better known and more abundant nutrients.

In terms of actual amounts of citric acid ingested and excreted, the men with an average intake of 22.49 gm. in the fruit juice excreted 0.399 ± 0.024 gm. "extra" in the urine; the women, with their intake increased by 21.29 gm., showed a mean rise in excretion of 0.468 ± 0.036 gm.

Despite the fact that the twenty-five subjects of these experiments were given from two to four times as much citric acid as those of Schuck, not one showed difficulty of efficient utilization even approaching that of her subject mentioned earlier, who excreted 1.294 gm. (16.6%) of the acid unchanged. The largest quantity of citric acid escaping oxidation in our series (case of M. C. U.) corresponds in actual amount to only three-fifths, and in percentage of the intake, to only one-fifth, of the comparable values for Schuck's "poorest" subject. The conclusion therefore seems warranted that cases of such

relatively poor utilization are seldom encountered, and that most persons are able to metabolize almost completely larger quantities of citric acid than they are apt to obtain through even very liberal consumption of citrus fruits.

Effect of grapefruit juice on urinary acidity. In each of the subjects observed, the pH of the 24-hour urine sample was higher on the day the juice was consumed than on the preceding control day. The average increase was 0.66 $\pm$ 0.044 pH unit for the entire group of twenty-five (0.67 for males; 0.64 for females).

Considering only the ten subjects whose diet (and hence their intake of acid- and base-forming elements) was the same, except for the grapefruit juice, on the control and experimental days, there appears a mean increase of 0.53 $\pm$ 0.11 unit over an average value of pH 6.00 on the control day. Further, excluding C. S. L. who, on the experimental day, took unaccustomed strenuous exercise and may for this reason have excreted extra lactic acid, the remaining nine best-controlled subjects show an increase in urinary pH of 0.58 $\pm$ 0.09, attributable to consumption of grapefruit juice.

It may possibly be argued that, though large intakes of citrus juice have a net alkalizing effect as shown in the 24-hour urine sample, there may, nevertheless, circulate for a short time sufficient extra acid citrate from the juice to produce a tendency toward increased acidity. However, the four short-time experiments briefly described above failed to yield evidence of such an effect, for no one of the successive samples of urine collected up to 22 hours after ingestion of the juice was more acid than the control sample collected the 2 hours before, and most of them were distinctly more alkaline.

The basal citric acid excretion

Effect of citrate intake. As might be expected from the ease with which the body was found to destroy almost completely the large amounts of citric acid in grapefruit juice, the variations in citric acid excretion among all individuals on low-citrate diets appear to bear little relation to the differences

in their citrate intakes. Thus, the Pearsonian coefficient, r, for the correlation between the intake and excretion of citric acid on the control day was $+ 0.086 \pm 0.198$ for the entire group; or, considering the sexes separately, $+ 0.156 \pm 0.325$ for the women and $+ 0.335 \pm 0.149$ for the men.

Even for the same individual, the increase of citric acid excretion when grapefruit juice is taken is not as dependent quantitatively on the increase of citric acid intake as might have been anticipated, r for this relationship being 0.410 ± 0.112 in the present series. Such a correlation coefficient, while probably "statistically significant", indicates that the two factors are associated only to a degree characterized as moderate by Chaddock ('25).

Relation to acid-base balance. In the experiments of Sherman, Mendel and Smith ('36 a) already cited, it was shown that for a given individual the excretion of citric acid tends to rise with an increase in urinary pH, and vice versa, whether the change in pH results from a shift in the articles of food consumed or through such means as the ingestion of sodium bicarbonate. In the present series, as pointed out above, the taking of large amounts of grapefruit juice in each case resulted both in an increase of citrate excretion and in a rise of urinary pH. That the extent of these changes is interdependent is clearly shown by the coefficient of $+ 0.640 \pm 0.118$ for the correlation between rise in urinary pH and rise in citrate excretion for a given individual. Indeed, the interesting observation that the degree of association is here marked, as contrasted with only a moderate positive correlation between citric acid intake and excretion (noted in the preceding paragraph) illustrates well the argument stressed earlier in this paper for regarding at least part of the increased citrate excretion as due to an acid-base shift per se.

However, as between different individuals, a consistent relation of urinary pH to basal citrate excretion was not found in the present series, r in the group of females being $- 0.334 \pm 0.296$, while for the males it was $+ 0.237 \pm 0.236$.

Sex difference. The basal excretion of citrate by the males averaged 0.403 gm. $\pm$ 0.021 while that of the females was

definitely higher, 0.643 ± 0.033 gm. Though we have no explanation for it, we are inclined to believe that this apparent sex difference is real.

SUMMARY

Twenty-five young adult subjects were found to utilize on the average 98% of the citric acid and citrates provided by very liberal (1800 cc. per day) consumption of grapefruit juice. Further evidences of a high tolerance toward citrus fruits were the absence of subjective symptoms and the finding in each case that consumption of grapefruit diminished the acidity of the 24-hour urine sample. Several additional studies in which a large (920 to 1675 cc.) single dose of grapefruit juice was taken and urine collections were made at short intervals showed a similarly high percentage utilization of the contained citric acid and a rise in urinary pH, i.e., a shift toward alkalinity, at all times up to 22 hours after ingestion of the juice.

We wish to express our thanks to Mrs. A. B. Rohrer, Mrs. Grace G. Stroup, and Dr. Oscar E. Lanford for their assistance with the analyses, and to the experimental subjects for their willing cooperation.

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THE DISTRIBUTION OF NICOTINIC ACID IN FOODS [1]

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The extensive use of nicotinic acid in the prevention and cure of pellagra makes it desirable to extend our knowledge about its distribution in foods. Information along this line has been rather meager because of the limitations of the methods of assay that have been employed. Bacharach ('41) has recently summarized most of the published data.

In general, the proposed microbiological methods for the determination of nicotinic acid have been limited in their application by relative inaccuracy, the necessity for clear extracts, and pathogenicity of the organisms. These difficulties have been largely eliminated in the microbiological method of Snell and Wright ('41) which has proven to be very satisfactory in our hands.

EXPERIMENTAL

The method is based on the response of Lactobacillus arabinosus 17-5 to nicotinic acid or to its amide. Acid production is plotted against known nicotinic acid concentration to obtain a standard curve. The nicotinic acid content of the unknown is obtained by interpolation on the standard curve. Acid production is proportional to nicotinic acid concentration from 0.04 to 0.20 μg. of nicotinic acid per tube.

A number of workers, in attempting to use the method of Snell and Wright, have experienced difficulty in obtaining low blanks. The assay of medium constituents has shown that casein, biotin concentrates, and cystine preparations may

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from General Mills, Inc.

be sources of nicotinic acid contamination.[2] Labco casein or acid-washed casein prepared according to the method of Arnold and Elvehjem ('38) give satisfactory blanks. The amount of casein used in the medium contributes from 0.7 cc. to 1.0 cc. to the blank titration. Extraction of the casein with alcohol reduces this amount to 0.5 cc.

The original procedure recommends incubation at 30°C. It has been found that incubation at 37°C. gives identical results.

TABLE 1

Comparison of various methods of extraction in the microbiological determination of nicotinic acid.

SAMPLE	METHOD OF EXTRACTION		
	H_2O	HCl	NaOH
	mg.%	*mg.%*	*mg.%*
Skim milk powder I	0.88	0.90	0.89
Liver concentrate 14600	114	112	112
Whole wheat I	4.9	6.9	6.8
Whole wheat II	4.5	6.1	6.1
Whole wheat III	4.0	5.1	5.3
Patent flour I	0.90	1.15	1.18
Patent flour II	1.07	.....	1.05
Patent flour III	1.10	.....	1.30
Dried baker's yeast I	40.0	.....	41.2
Dried grass concentrate	16.0	.....	15.5
Dried cows' rumen contents	6.3	.....	6.2
Dried fried ham 141	.....	18.0	19.5
Dried beef liver 98	.....	43.0	42.3
Dried beef spleen 76	.....	18.5	19.7

Preparation of sample

The stability of nicotinic acid makes it possible to use rather severe treatments in order to obtain good extraction. The effectiveness of various methods of extraction is shown in table 1. For most materials, water extraction is satisfactory. However, in the case of cereals, alkaline or acid treatment is necessary for complete extraction. Water extraction was carried out by suspending from 0.1 to 0.5 gm. of finely ground

[2] Cystine hydrochloride obtained from the Eastman Kodak Co., Rochester, New York, and biotin concentrates from the S.M.A. Corp., Chagrin Falls, Ohio, have been found to be satisfactory. At present crystalline biotin is available and should, of course, be used in preference to impure biotin concentrates.

material in 75 cc. of water, autoclaving for 20 minutes at 15 pounds pressure, and diluting to the desired volume. Treatment with HCl involved heating from 0.1 gm. to 0.5 gm. of material on a steam bath for 1 hour with 25 cc. of 5 N HCl, neutralizing with NaOH (brom thymol blue is used as an outside indicator), and diluting to a convenient volume. The NaOH treatment was carried out as follows: From 0.1 gm. to 0.5 gm. of finely ground material is suspended in approximately 75 cc. of water, 20 cc. of 8% NaOH are added, and the suspension is autoclaved at 15 pounds pressure for 20 minutes. It is then neutralized with HCl and diluted to a convenient volume.

TABLE 2

Recovery of added nicotinic acid.

SAMPLE	NICOTINIC ACID FOUND IN ORIGINAL SAMPLE	NICOTINIC ACID ADDED	TOTAL NICOTINIC ACID FOUND	PER CENT RECOVERY
	μg./gm.	*μg./gm.*	*μg./gm.*	
Whole wheat	49	50	100	102
Dried baker's yeast I	400	500	880	96
Patent flour IV	12	25	38	104
Yellow corn	24	50	75	102
Beef kidney 126	315	250	560	98
Evaporated milk I	1.8	5	7.2	108
Beef liver 98	455	500	904	90

If a sample of whole wheat is being assayed, a 0.2 gm. sample containing approximately 12 μg. of nicotinic acid is diluted to a final volume of 250 cc. Each cubic centimeter of solution then contains approximately 0.05 μg. of nicotinic acid and aliquots from 1 cc. to 4 cc. fall in the range of the assay.

In these studies, fresh fruits and vegetables were homogenized in a Waring Blendor, and a water suspension was then treated with NaOH as outlined above.

Recovery of nicotinic acid when added to a wide variety of materials ranged from 90% to 110%, as shown in table 2. These results substantiate the conclusion of Snell and Wright ('41) that ordinary materials do not contain interfering amounts of inhibitory or stimulatory substances.

In order to study the assay of food mixtures, various combinations of liver, corn, dried grass, yeast, rice, skim milk powder, wheat and bananas have been assayed. As shown in table 3, the values obtained checked the calculated values based on the assay of each component.

TABLE 3

The assay of food mixtures for nicotinic acid.

MIXTURE I	NICOTINIC ACID μg.
Banana 0.7 gm.	4.5
Pork liver 0.1 gm.	54.0
Corn meal 1.0 gm.	10.3
Skim milk powder 0.5 gm.	5.0
	73.8
Found	72.0

MIXTURE II	
Baker's yeast 0.1 gm.	40.0
Brown rice 0.5 gm.	34.5
Whole wheat 0.5 gm.	30.5
Dried grass 0.5 gm.	36.0
	141.0
Found	150.0

MIXTURE III	NICOTINIC ACID μg.
Whole wheat 0.5 gm.	30.5
Skim milk powder 0.5 gm.	5.0
Dried grass 0.5 gm.	36.0
	71.5
Found	75.0

MIXTURE IV	
Pork liver 0.1 gm.	54.0
Corn meal 1.0 gm.	10.3
Dried grass 0.5 gm.	36.0
Yeast 0.1 gm.	40.0
Brown rice 0.5 gm.	34.5
Skim milk powder 0.5 gm.	5.0
Whole wheat 0.5 gm.	30.5
Banana 0.7 gm.	4.5
	214.8
Found	217.0

TABLE 4

Comparison of chemical and microbiological assays of dried meats for nicotinic acid.

SAMPLE	CHEMICAL ASSAY	MICROBIOLOGICAL ASSAY
	mg.%	*mg.%*
Pork kidney 62	47.9	45.5
Pork loin 74	21.4	23.5
Pork ham 95	27.0	28.0
Fried beef liver 121	53.3	38.0
Veal liver 70	50.8	41.0
Veal hindquarter 134	31.4	29.0
Beef pancreas 64	18.0	15.7
Light chicken 68	29.7	29.0

TABLE 5

Nicotinic acid content of fruits, vegetables, cereals and miscellaneous materials.

TABLE 5

Nicotinic acid content of fruits, vegetables, cereals and miscellaneous materials.

FRUITS AND VEGETABLES		
Sample	Fresh	Dry
	mg.%	*mg.%*
Apple (peeled)	0.50	3.54
Apple peelings	1.13	7.15
Banana	0.61	2.90
Beans, wax	0.76	7.45
Beans, green	0.64	6.09
Beans, kidney		2.82
Beans, lima		1.83
Cabbage	0.29	4.46
Carrot	1.47	14.0
Cauliflower	0.57	7.13
Cranberries	0.13	1.23
Cucumber	0.32	8.0
Dates	2.18	
Grapes (Thompson seedless)	0.28	1.84
Kohlrabi	0.27	3.86
Onion	0.10	0.77
Peach (peeled)	0.95	7.92
Pear (peeled)	0.14	1.15
Pear peelings	0.34	1.42
Potato, white (peeled)	1.18	5.54
Potato, white, peelings	1.01	5.05
Potato, sweet (peeled)	1.29	4.53
Potato, sweet (peelings)	1.71	4.97
Plum (peeled)	0.56	3.86
Plum peelings	0.67	3.52
Raisins	0.63	
Red beet	0.64	4.74
Soybean flour		2.40
Spinach	0.72	7.65
Tomato (peeled)	0.58	16.5
Tomato peelings	0.76	9.50
Yam (peeled)	0.67	2.20
Yam peelings	1.51	4.80
	mg. per 100 cc.	
Grapefruit juice	0.21	
Lemon juice	0.08	
Lime juice	0.09	
Orange juice	0.22	
Tomato juice	0.10	

CEREALS	
Sample	
	mg.%
Oats — Sample I	1.13
Oats — Sample II	1.60
Red barley	4.70
Pearled barley	2.75
Buckwheat	4.40
Polished rice	0.90
Brown rice	6.90
Rice polishings	96.6
Rye — Sample I	0.90
Rye — Sample II	1.03
Rye — Sample III	1.29
Yellow corn (8 samples)	1.56–2.60
Yellow corn meal — Sample I	1.03
Yellow corn meal — Sample II	1.08
White corn meal	1.76
Whole wheat	5.0–7.0
Milling fractions of wheat	
Patent flour	0.80
Fancy first clear flour	1.68
Second clear flour	5.55
Clean shorts	8.44
Wheat feed and screenings	19.2
Bran — Sample I	25.7
Bran — Sample II	28.7
Bran — Sample III	40.0
Seminola	2.01
Farina	0.98
Standard middlings and screenings	13.0
Flour middlings and screenings	9.2
Durum middlings	17.7
Wheat middlings — Sample II	10.6
Wheat germ — Sample I	3.4
Wheat germ — Sample II	4.7
Defatted wheat germ	6.6
Wheat gluten	2.5

MISCELLANEOUS MATERIALS	
Sample	
	mg.%
Corn silage	1.5
Linseed oil meal	4.3
Timothy hay	2.3
Alfalfa meal	3.9
Malt sprouts	5.6
Dried grass	7.2
Whole milk	0.08
Evaporated milk I	0.18
Acidophilus milk	0.08
Skim milk powder	0.89
Human milk	0.26
Macaroni	2.10
Peanut butter	18.6
Egg white (hard boiled)	0.076
Egg yolk (hard boiled)	0.035
Paprika	1.38
Canned shrimp	0.78
Canned oyster	0.66
Canned clam	1.08
Brewer's yeast	40.0–60.0
Baker's yeast	40.0–50.0
Dried blood	2.7
Breads	*Fresh mg.%*
White bread I	0.68
White bread II	0.95
White bread III	0.66
Enriched white bread	1.51
Milk bread	0.92
Whole wheat blend I	1.80
Whole wheat blend II	1.70
100% whole wheat bread	2.88
Potato bread	0.81
Soybean bread	1.35

DISCUSSION

In general, values obtained by microbiological assay agree quite well with values obtained by chemical analysis. In the assay of meats, excellent agreement has been obtained, as shown in table 4. The values for the chemical assay are taken from the report of McIntire, Waisman, Henderson and Elvehjem ('41). The values for kidney, muscle and pancreas agree very well, the only marked discrepancy being in the case of liver. The microbiological assay of liver gives results that are somewhat lower.

Most fruits and vegetables are not very good sources of nicotinic acid (table 5). On a dry weight basis, carrots and tomatoes are the best of this group. Meats in general, particularly the organs, yeast and peanut butter are excellent sources. Milk is very low in nicotinic acid content, containing about 0.08 mg. per cent. Eggs are also a very poor source.

TABLE 6

The nicotinic acid content of soft wheat flours obtained from various sections of the United States.[1]

STATE FROM WHICH SAMPLE WAS OBTAINED	PATENT FLOUR	CLEAR FLOUR	CUT OFF FLOUR
	mg.%	*mg.%*	*mg.%*
Michigan	0.80	1.11	
Tennessee	0.79	1.00	
Missouri	0.82	1.18	
North Carolina	0.76	1.02	
Kentucky	0.75		1.43
Tennessee	0.84		1.30

[1] The authors wish to express their thanks to Mr. W. H. Strowd, National Soft Wheat Millers Association, Nashville, Tennessee, for providing the flour samples listed in this table.

Table 5 shows that whole cereals vary considerably in their nicotinic acid content. Corn, oats, and rye are all quite low; buckwheat, barley, and whole wheat contain from 4.5 to 7.0 mg. per cent; white flour contains about 1.0 mg. per cent.

The analysis of various milling fractions of wheat (table 5) indicates that most of the vitamin is contained in the bran and middling fraction. Patent flour is low, but some nicotinic acid is retained in the coarser clear and cutoff flours (table 6).

SUMMARY

A study has been made of the application of the microbiological method of Snell and Wright ('41) to the assay of foods for nicotinic acid. The nicotinic acid content of a variety of foods is reported.

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